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(54) Title: METHOD OF SCREENING FOR POTENTIAL ANTI-METASTATIC AND ANTI-INFLAMMATORY AGENTS USING MAMMALIAN HEPARANASE AS A PROBE

(57) Abstract

Qualitative and quantitative methods of testing an agent for its potential at inhibiting glycosidase catalytic activity, the methods including the steps of interacting a glycosidase enzyme with a glycosidase substrate in a presence of the agent and qualitatively or quantitatively evaluating an effect of the agent on the catalytic activity of the glycosidase enzyme toward the glycosidase substrate. Preferably the glycosidase enzyme is a heparanase enzyme and the glycosidase substrate is, respectively, a heparanase substrate.

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METHOD OF SCREENING FOR POTENTIAL ANTI- METASTATIC AND ANTI-INFLAMMATORY AGENTS USING MAMMALIAN HEPARANASE AS A PROBE

5   ## FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to novel qualitative and quantitative glycosidases catalytic activity assays. More particularly, the present invention relates to a method of screening for potential anti-metastatic and anti-inflammatory agents and, most particularly, to a method of screening
10   for potential anti-metastatic and anti-inflammatory agents using mammalian heparanase, preferably purified human recombinant heparanase, as a probe.

heparan sulfate proteoglycans (HSPGs): HSPGs are ubiquitous macromolecules associated with the cell surface and extracellular matrix (ECM) of a wide range of cells of vertebrate and invertebrate tissues (1-5).
15   The basic HSPG structure consists of a protein core to which several linear heparan sulfate chains are covalently attached. The polysaccharide chains are typically composed of repeating hexuronic and D-glucosamine disaccharide units that are substituted to a varying extent with N- and O-linked sulfate moieties and N-linked acetyl groups (1-5). Studies on the
20   involvement of ECM molecules in cell attachment, growth and differentiation revealed a central role of HSPGs in embryonic morphogenesis, angiogenesis, metastasis, neurite outgrowth and tissue repair (1-5). The heparan sulfate (HS) chains, which are unique in their ability to bind a multitude of proteins, ensure that a wide variety of effector
25   molecules cling to the cell surface (4-6). HSPGs are also prominent components of blood vessels (3). In large vessels they are concentrated mostly in the intima and inner media, whereas in capillaries they are found mainly in the subendothelial basement membrane where they support proliferating and migrating endothelial cells and stabilize the structure of
30   the capillary wall. The ability of HSPGs to interact with ECM macromolecules such as collagen, laminin and fibronectin, and with different attachment sites on plasma membranes suggests a key role for this proteoglycan in the self-assembly and insolubility of ECM components, as well as in cell adhesion and locomotion. Cleavage of HS may therefore
35   result in disassembly of the subendothelial ECM and hence may play a decisive role in extravasation of normal and malignant blood-borne cells (7-9). HS catabolism is observed in inflammation, wound repair, diabetes, and cancer metastasis, suggesting that enzymes which degrade HS play important roles in pathologic processes.

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Involvement of heparanase in tumor cell invasion and metastasis:
Circulating tumor cells arrested in the capillary beds of different organs
must invade the endothelial cell lining and degrade its underlying basement
membrane (BM) in order to escape into the extravascular tissue(s) where
they establish metastasis (10). Several cellular enzymes (e.g., collagenase
IV, plasminogen activator, cathepsin B, elastase) are thought to be involved
in degradation of the BM (10). Among these enzymes is an endo-β-D-
glucuronidase (heparanase) that cleaves HS at specific intrachain sites (7, 9,
11-12). Expression of a HS degrading heparanase was found to correlate
with the metastatic potential at mouse lymphoma (11), fibrosarcoma and
melanoma (9) cells. The same is true for human breast, bladder and
prostate carcinoma cells (see U.S. Pat. application 09/109,386, and
PCT/US98/17954, which are incorporated by reference as if fully set forth
herein). Moreover, elevated levels of heparanase were detected in sera (9)
and urine (U.S. Pat. application 09/071.739; PCT/US99/09255) of
metastatic tumor bearing animals and cancer patients and in tumor biopsies
(12). Treatment of experimental animals with heparanase alternative
substrates and inhibitor (e.g., non-anticoagulant species of low MW
heparin, laminarin sulfate) markedly reduced (> 90 %) the incidence of lung
metastases induced by B16 melanoma, Lewis lung carcinoma and
mammary adenocarcinoma cells (8, 9, 13). indicating that heparanase
inhibitors may be applied to inhibit tumor cell invasion and metastasis.

Our studies on the control of tumor progression by its local
environment, focus on the interaction of cells with the extracellular matrix
(ECM) produced by cultured corneal and vascular endothelial cells (EC)
(14, 15). This ECM closely resembles the subendothelium *in vivo* in its
morphological appearance and molecular composition. It contains
collagens (mostly type III and IV, with smaller amounts of types I and V),
proteoglycans (mostly heparan sulfate- and dermatan sulfate- proteoglycans,
with smaller amounts of chondroitin sulfate proteoglycans), laminin,
fibronectin, entactin and elastin (13, 14). The ability of cells to degrade HS
in the ECM was studied by allowing cells to interact with a metabolically
sulfate labeled ECM, followed by gel filtration (Sepharose 6B) analysis of
degradation products released into the culture medium (11). While intact
HSPG are eluted next to the void volume of the column (Kav < 0.2, Mr of
about 0.5×10^6), labeled degradation fragments of HS side chains are eluted
more toward the Vt of the column ($0.5 <$ kav < 0.8, Mr of about $5\text{-}7 \times 10^3$)
(11). Compounds which efficiently inhibit the ability of heparanase to

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degrade the above-described naturally produced basement membrane-like substrate, were also found to inhibit experimental metastasis in mice and rats (8, 9, 13, 33). A reliable *in vitro* screening system for heparanase inhibiting compounds may hence be applied to identify and develop potent anti-metastatic drugs.

Possible involvement of heparanase in tumor angiogenesis: We have previously demonstrated that heparanase may not only function in cell migration and invasion, but may also elicit an indirect neovascular response (15). Our results suggest that the ECM HSPGs provide a natural storage depot for βFGF and possibly other heparin-binding growth promoting factors. Heparanase mediated release of active βFGF from its storage within ECM may therefore provide a novel mechanism for induction of neovascularization in normal and pathological situations (6, 18).

Expression of heparanase by cells of the immune system: Heparanase catalytic activity correlates with the ability of activated cells of the immune system to leave the circulation and elicit both inflammatory and autoimmune responses. Interaction of platelets, granulocytes, T and B lymphocytes, macrophages and mast cells with the subendothelial ECM is associated with degradation of heparan sulfate (HS) by heparanase catalytic activity (7). The enzyme is released from intracellular compartments (e.g., lysosomes, specific granules) in response to various activation signals (e.g., thrombin, calcium ionophore, immune complexes, antigens, mitogens), suggesting its regulated involvement and presence in inflammatory sites and autoimmune lesions. Heparan sulfate degrading enzymes released by platelets and macrophages are likely to be present in atherosclerotic lesions (16). Treatment of experimental animals with heparanase alternative substrates (e.g., non-anticoagulant species of low molecular weight heparin) markedly reduced the incidence of experimental autoimmune encephalomyelitis (EAE), adjuvant arthritis and graft rejection (7, 17) in experimental animals, indicating that heparanase inhibitors may be applied to inhibit autoimmune and inflammatory diseases (7, 17). A reliable *in vitro* screening system for heparanase inhibiting compounds may hence be applied to identify and develop non-toxic anti-inflammatory drugs for the treatment of multiple sclerosis and other inflammatory diseases.

Cloning and expression of the heparanase gene: A purified fraction of heparanase isolated from human hepatoma cells was subjected to tryptic digestion. Peptides were separated by high pressure liquid chromatography and micro sequenced. The sequence of one of the peptides

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was used to screen data bases for homology to the corresponding back translated DNA sequence. This procedure led to the identification of a clone containing an insert of 1020 base pairs (bp) which included an open reading frame of 963 bp followed by 27 bp of 3' untranslated region and a poly A tail. The new gene was designated *hpa*. Cloning of the missing 5' end of *hpa* was performed by PCR amplification of DNA from placenta cDNA composite. The entire heparanase cDNA was designated p*hpa*. The joined cDNA fragment contained an open reading frame which encodes a polypeptide of 543 amino acids with a calculated molecular weight of 61,192 daltons. Cloning an extended 5' sequence was enabled from the human SK-hep1 cell line by PCR amplification using the Marathon RACE system. The 5' extended sequence of the SK-hep1 *hpa* cDNA was assembled with the sequence of the *hpa* cDNA isolated from human placenta. The assembled sequence contained an open reading frame which encodes a polypeptide of 592 amino acids with a calculated molecular weight of 66,407 daltons. The cloning procedures are described in length in U.S. Pat. application Nos. 08/922,170; 09/109,386; and 09/258,892; and PCT/US98/17954, all of which are incorporated herein by reference.

The ability of the *hpa* gene product to catalyze degradation of heparan sulfate (HS) *in vitro* was examined by expressing the entire open reading frame of *hpa* in High five and Sf21 insect cells, and the mammalian human 293 embryonic kidney cell line expression systems. Extracts of infected cells were assayed for heparanase catalytic activity. For this purpose, cell lysates were incubated with sulfate labeled, ECM-derived HSPG (peak I), followed by gel filtration analysis (Sepharose 6B) of the reaction mixture. While the substrate alone consisted of high molecular weight material, incubation of the HSPG substrate with lysates of cells infected with *hpa* containing virus resulted in a complete conversion of the high molecular weight substrate into low molecular weight labeled heparan sulfate degradation fragments (U.S. Pat. application Nos. 08/922,170; 09/109,386; and 09/258,892; and PCT/US98/17954).

In subsequent experiments, the labeled HSPG substrate was incubated with the culture medium of infected High Five and Sf21 cells. Heparanase catalytic activity, reflected by the conversion of the high molecular weight HSPG substrate into low molecular weight HS degradation fragments, was found in the culture medium of cells infected with the pF*hpa* virus, but not the control pF1 virus.

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Altogether, these results indicate that the heparanase enzyme is expressed in an active form by cells infected with Baculovirus or mammalian expression vectors containing the newly identified human *hpa* gene.

In other experiments, we have demonstrated that the heparanase enzyme expressed by cells infected with the pF*hpa* virus is capable of degrading HS complexed to other macromolecular constituents (e.g., fibronectin, laminin, collagen) present in a naturally produced intact ECM, in a manner similar to that reported for highly metastatic tumor cells or activated cells of the immune system (7, 8)

Purification of the recombinant heparanase enzyme: Sf21 insect cells were infected with pF*hpa* virus and the culture medium was applied onto a heparin-Sepharose column. Fractions were eluted with a salt gradient (0.35-2.0 M NaCl) and tested for heparanase catalytic activity and protein profile (SDS/PAGE followed by silver staining). Heparanase catalytic activity correlated with the appearance of a about 63 kDa protein band in fractions 19-24, consistent with the expected molecular weight of the *hpa* gene product. Active fractions eluted from heparin-Sepharose were pooled, concentrated and applied onto a Superdex 75 FPLC gel filtration column. Aliquots of each fraction were tested for heparanase catalytic activity and protein profile. A correlation was found between the appearance of a major protein (approximate molecular weight of 63 kDa) in fractions 4-7 and heparanase catalytic activity. This protein was not present in medium conditioned by control non-infected Sf21 cells subjected to the same purification protocol. Recently, an additional purification protocol was applied, using a single step chromatography with source-S ion exchange column. This purification resulted in a purified protein to a degree of 90 %. Further details concerning theses purification procedure are disclosed in U.S. Pat. application Nos. 09/071,739 and 09/071,618; and PCT/US99/09255, all are incorporated by reference as if fully set forth herein.

Recombinant heparanase for screening purposes: Research aimed at identifying and developing inhibitors of heparanase catalytic activity has been handicapped by the lack of a consistent and constant source of a purified and highly active heparanase enzyme and of a reliable screening system. Our recent cloning, expression and purification of the human heparanase-encoding gene offer, for the first time, a most appropriate and reliable source of active recombinant enzyme for screening of anti-

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heparanase antibodies and compounds which may inhibit the enzyme and hence be applied to identify and develop drugs that may inhibit tumor metastasis, autoimmune
and inflammatory diseases.

Screening for specific inhibitors using a combinatorial library: A new approach aimed at rational drug discovery was recently developed for screening for specific biological activities. According to the new approach, a large library of chemically diversed molecules are screened for the desired biological activity. The new approach has become an effective and hence important tool for discovery of new drugs. The new approach is based on "combinatorial" synthesis of a diverse set of molecules in which several components predicted to be associated with the desired biological activity are systematically varied. The advantage of a combinatorial library over the alternative use of natural extracts for screening for desired biologically active compounds is that all the components comprising the library are known in advance (50).

In combinatorial screening, the number of hits discovered is proportional to the number of molecules tested. This is true even when knowledge concerning the target is unavailable. The large number of compounds, which may reach thousands of compounds tested per day. can only be screened, provided that a suitable assay involving a high throughput screening technique, in which laboratory automation and robotics may be applied, exists.

Prior art heparanase catalytic activity assays: Several methods for determining heparanase catalytic activity have been developed throughout the years. All of the different methods are based on radiolabeling of a substrate (either *in vitro* or metabolically, as described above) and analysis of its degradation products released due to heparanase catalytic activity. These prior art methods suffer several disadvantages and limitations as follows.

First, the measurement of catalytic activity is qualitative and not quantitative. This is due to the following reasons (i) the radioactive labeling is not spread evenly along the substrate chain, therefore, radioactivity may not correlate precisely with activity; (ii) since heparanase substrates are long substrate chains, a released product can be, in fact, a substrate of heparanase, however while executing any of the prior art methods, cleavage events of released products are not monitorable. Moreover, multiple cleavage events of small portions of the substrate chain are

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indistinguishable from fewer cleavage events, yet of longer substrate chains. Thus, not all, and in many cases, depending on the substrate chain length, not even most, of the cleavage events catalyzed by the enzyme are detectable, thereby affecting the linearity of the assay.

5   Second, the prior art methods are cumbersome, time-consuming and do not allow activity determination of a large number of samples simultaneously. In most cases, both preparation of the radiolabeled substrate and separation of the degradation products from the uncleaved substrate involve long and complex procedures and handling with radioactive material which calls strict safety procedures.

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        Third, all of the prior art methods for determining heparanase catalytic activity involve modification of the substrate by either iodination at glucosamine residues, or either O- or N-acetylation of the partially de-N-sulfated substrate. Such procedures may result in masking heparanase cleavage sites, or alternatively creating new heparanase sites.

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        The different prior art methods also have specific disadvantages specifically associated with each of which. Some methods involve biosynthetic radiolabeling of ECM associated HSPG and detection of HS chain degradation by gel filtration analysis of the radiolabeled material

20   released from the labeled ECM (7, 37). In these assays, detection of the products requires a synergistic activity of proteases and heparanase. Protease is required to expose HS chains to cleavage by heparanase.

        Other methods involve immobilizing chemically or biosynthetically radiolabeled heparanase substrate chains (38, 39, 40). The main

25   disadvantage of these methods is that the immobilized substrate may be less accessible to the enzyme.

        In the heparanase catalytic activity assay recently developed by Freeman and Parish (41) the products are separated from the substrate by binding to chicken histidine-rich glycoprotein (cHRG) sepharose. In this

30   method only the lowest molecular weight products that lose the ability to bind to cHRG sepharose are detectable, while other, longer, products bind to the column with the substrate and are therefore excluded.

        The mechanism by which heparanase operates on its substrate is still unknown and it is possible that some chains may first be cleaved to longer

35   chains and then further be degraded to smaller fragments, while other chains may be directly cleaved at the end of thereof to form small fragments. The method by Freeman and Parish, therefore. fails to detect all of the cleavage

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products and therefore, like all of the other prior art methods for assaying heparanase catalytic activity, it is qualitative rather than quantitative.

The lack of a quantitative heparanase catalytic activity assay combined with the time and labor required to analyze a single sample using the qualitative prior art methods highlights the need for a rapid quantitative heparanase catalytic activity assay capable of assaying a large number of samples simultaneously.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a method of testing an agent for its potential at inhibiting heparanase catalytic activity. the method comprising the steps of interacting a recombinant heparanase enzyme with a heparanase substrate in a presence of the agent and evaluating an effect of the agent on the catalytic activity of the recombinant heparanase enzyme toward the heparanase substrate.

According to another aspect of the present invention there is provided a method of screening for agents inhibiting heparanase catalytic activity and hence potentially inhibiting tumor metastasis, autoimmunity and inflammatory diseases, the method comprising the steps of. in individual reactions, interacting a recombinant heparanase enzyme with a heparanase substrate in a presence of each of the agents and evaluating an effect of each of the agents on the catalytic activity of the recombinant heparanase enzyme toward the heparanase substrate.

According to yet another aspect of the present invention there is provided a quantitative method of testing an agent for its potential at inhibiting glycosidase catalytic activity, the method comprising the steps of interacting a glycosidase enzyme with a glycosidase substrate in a presence of the agent and quantitatively evaluating an effect of the agent on the catalytic activity of the glycosidase enzyme toward the glycosidase substrate. Preferably the glycosidase enzyme is a heparanase enzyme and the glycosidase substrate is, respectively, a heparanase substrate.

According to still another aspect of the present invention there is provided a method of screening for agents inhibiting heparanase catalytic activity and hence potentially inhibiting tumor metastasis, autoimmunity and inflammatory diseases, the method comprising the steps of, in individual reactions, interacting a heparanase enzyme with a heparanase substrate in a presence of each of the agents and quantitatively evaluating an

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effect of each of the agents on the catalytic activity of the heparanase enzyme toward the heparanase substrate.

According to still another aspect of the present invention there is provided a method of testing heparanase catalytic activity, the method comprising the steps of interacting a recombinant heparanase enzyme with a heparanase substrate and evaluating the catalytic activity of the recombinant heparanase enzyme toward the heparanase substrate.

According to still another aspect of the present invention there is provided a quantitative method of testing glycosidase catalytic activity, the method comprising the steps of interacting a glycosidase enzyme with a glycosidase substrate and quantitatively evaluating the catalytic activity of the glycosidase enzyme toward the glycosidase substrate.

According to further features in preferred embodiments of the invention described below, the glycosidase or heparanase (including recombinant heparanase) are each independently of a human origin.

According to still further features in the described preferred embodiments the heparanase is a natural heparanase.

According to still further features in the described preferred embodiments the recombinant heparanase is expressed in an expression system selected from the group consisting of an insect cell expression system, a mammalian cell expression system and a yeast cell expression system.

According to still further features in the described preferred embodiments the substrate is radiolabeled.

According to still further features in the described preferred embodiments the radiolabeled substrate is radiolabeled by a radiolabeling method selected from the group consisting of *in vitro* radiolabeling and metabolically radiolabeling.

According to still further features in the described preferred embodiments the substrate is an extracellular matrix or a portion thereof.

According to still further features in the described preferred embodiments the substrate includes macromolecules associated with the extracellular matrix.

According to still further features in the described preferred embodiments the macromolecules include heparan sulfate proteoglycans. In other words, the substrate preferably includes extracellular matrix-derived soluble heparan sulfate proteoglycans.

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According to still further features in the described preferred embodiments the substrate is selected from the group consisting of heparan sulfate, heparin, heparin-sepharose, and derivatives thereof.

According to still further features in the described preferred embodiments the substrate is immobilized to a solid support, e.g., heparin-sepharose.

According to still further features in the described preferred embodiments the agent or agents include an anti-heparanase antibody.

According to still further features in the described preferred embodiments the agent or agents include a naturally occurring agent.

According to still further features in the described preferred embodiments the agent or agents include a synthetic agent or a library of synthetic agents.

According to still further features in the described preferred embodiments the synthetic agent is one of a plurality of agents belonging to a combinatorial library of similar agents.

According to still further features in the described preferred embodiments evaluating the effect of the agent or agents on the catalytic activity of the recombinant heparanase enzyme toward the heparanase substrate is effected by a size separation assay adapted for detection of degradation products of the heparanase substrate.

According to still further features in the described preferred embodiments the size separation assay is selected from the group consisting of gel electrophoresis and column chromatography.

According to still further features in the described preferred embodiments quantitatively evaluating the effect the catalytic activity of the glycosidase enzyme toward the glycosidase substrate and the effect of the agent or agents thereupon is effected by an assay adapted for detection of reducing moieties associated with degradation products of the glycosidase substrate.

According to still further features in the described preferred embodiments quantitatively evaluating the catalytic activity of the glycosidase enzyme toward the glycosidase substrate and the effect of the agent or agents thereupon is effected by a colorimetric assay.

According to still further features in the described preferred embodiments quantitatively evaluating the catalytic activity of the heparanase enzyme toward the heparanase substrate and the effect of the agent or agents thereupon is effected by an assay adapted for detection of

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reducing moieties associated with degradation products of the heparanase substrate.

According to still further features in the described preferred embodiments the assay is a reducing sugar assay.

According to still further features in the described preferred embodiments the colorimetric assay is a tetrazolium blue assay in which tetrazolium blue is reduced to a soluble colored formazan salt.

According to still further features in the described preferred embodiments the method behaves substantially linearly in a range of enzyme concentration.

According to still further features in the described preferred embodiments the method behaves substantially linearly in a range of time.

According to still further features in the described preferred embodiments the colorimetric assay is selected from the group consisting of carbazole assay and methylene blue assay.

The present invention successfully addresses the shortcomings of the presently known configurations by providing in one aspect, novel colorimetric qualitative and quantitative heparanase (and other glycosidases) activity assays. The qualitative assays are base on colorimetric detection of uronic (e.g., iduronic) acids in a complete hydrolyzate of the products released by heparanase catalytic activity acting on an immobilized substrate (e.g., heparin-sepharose), or on colorimetric detection of sulfated glycosaminoglycans similarly released from the immobilized substrate. It is therefore easier to implement as compared to the radioactive methods which call for size separation and like the radioactive methods it is applicable for activity determinations of crude enzyme extracts, as well as purified enzyme.

The quantitative assay is based on detection of newly formed reducing ends produced due to cleavage of polysaccharides, such as, but not limited to, heparin or heparan sulfate by glycosidases. This assay therefore detects every single cleavage event and can be used to extract activity catalytic constants of the enzyme and of inhibitors/activators thereof. The new assays allow for efficient screening of a large number of different agents simultaneously for their inhibitory effects on the enzyme. More specifically, these assays can be used to screen for potential inhibitors of heparanase and other glycosidases, by, for example, using recombinant heparanase combined with heparan sulfate or heparin as a substrate.

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BRIEF DESCRIPTION OF THE DRAWINGS

The invention herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIG. 1 demonstrates polyacrylamide gel analysis of heparanase catalytic activity. Heparin and heparan sulfate were incubated (24 hours, 37 °C, pH 5.4) with human recombinant heparanase enzyme. Following incubation, degradation products were fractionated on an acrylamide gel and were stained with methylene blue. Lane A - heparin control; lane B - heparin incubated with the recombinant heparanase; lane C - heparan sulfate control; lane D - heparan sulfate incubated with the recombinant heparanase.

FIGs. 2a-b demonstrate apparent inhibition of heparanase catalytic activity by heparin. Recombinant heparanase was incubated (24 hours, 37 °C, pH 6.6) with sulfate labeled ECM (Figure 2a) or sulfate labeled ECM-derived soluble HSPG (peak I material, Figure 2b, ◊), in the absence (peak II, ●) and presence (peak I, , △) of 5 μg/ml heparin. Sulfate labeled degradation products were analyzed by gel filtration over Sepharose 6B.

FIG. 3 demonstrates the susceptibility of Peak II material to nitrous acid deamination. Sulfate labeled ECM-derived soluble HSPG (peak I material) was incubated (37 °C, 2 hours, pH 6.2) with recombinant heparanase in a total volume of 1 ml. The reaction mixture (0.5 ml) was subjected to nitrous acid deamination (0.24 M $NaNO_3$ in 1.8 M acetic acid, 80 minutes, 24 °C). The substrate (■; SS 68), untreated (△; +Re-h#2 Hep5, 60'), and nitrous acid treated (O +Re-h#2 Hep5 + N.Acid) peak II material were subjected to gel filtration over Sepharose 6B columns.

FIG. 4 demonstrates inhibition of heparanase by heparin derived oligosaccharides of varying sizes. Sulfate labeled ECM was incubated (37 °C, 24 hours, pH 6.2) with recombinant heparanase in the absence (control) and presence of 1 μg/ml heparin fragment (84588-Fragmin) or size homogenous tetra and tetradeca-saccharides (90420-4 units and 19420-14 units, respectively) prepared by nitrous acid depolymerization of heparin. Sulfate labeled material released into the incubation medium was analyzed by gel filtration over Sepharose 6B columns.

FIG. 5 demonstrates inhibition of heparanase by chemically modified species of heparin fragment. Sulfate labeled ECM was incubated (37 °C, 24 hours, pH 6.2) with recombinant heparanase in the absence (control) or presence of 1 μg/ml heparin fragment (84588-Fragmin), totally desulfated (N-, O-) heparin fragment (70334-N/O) and species of heparin fragment in

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which the N-sulfates were substituted with acetyl (70421, N-acetyl) or hexanoyl (70901, N-hexanoyl) groups. Sulfate labeled material released into the incubation medium was analyzed by gel filtration over Sepharose 6B.

FIGs. 6a-c demonstrate inhibition of heparanase by synthetic polyanionic heparin-mimicking compounds. Sulfate labeled ECM-derived soluble HSPG (peak I material, ■) was incubated (37 °C, 24 hours, pH 6.2) with recombinant heparanase in the absence (●) or presence of 1, 5, or 10 μg/ml heparin (Figure 6a); compound P-97100 (Figure 6b); or compound RG-13577 (Figure 6c, O; ■; D, respectively). Sulfate labeled degradation products were analyzed by gel filtration over Sepharose 6B.

FIGs. 7a-b demonstrate inhibition of heparanase by non-sulfated and sulfated heparin-mimicking compounds. Sulfate labeled ECM-derived soluble HSPG (peak I material) was incubated (37 °C, 24 hours, pH 6.2) with recombinant heparanase in the absence (control) or presence of 0.1, 0.5, or 1 μg/ml compound P-97100 (Figure 7a); or compound AV-1/9-1 (Figure 7b). Sulfate labeled degradation products were analyzed by gel filtration over Sepharose 6B.

FIG. 8 demonstrates polyacrylamide gel analysis of heparanase catalytic activity with heparin-sepharose as a substrate. Heparin-sepharose was incubated (37 °C, 24 hours, pH 5.4) in the presence or absence of recombinant heparanase. The products were analyzed by fractionation on a native acrylamide gel stained with methylene blue. Lane A - heparin-sepharose control. Lane B - heparin-sepharose incubated with the recombinant heparanase. Heparanase catalytic activity may be visualized as a sift in the mobility of the products as compared to the substrate.

FIGs. 9a-b demonstrate determination of recombinant heparanase catalytic activity expressed by insect cells, yeast cells and mammalian cells, using the immobilized heparin and the carbazole or the dimethylmethylene blue colorimetric assays. Heparin-sepharose was incubated (37 °C , 17 hours, pH 5.4) with increasing amounts of extracts of mammalian cell expressing human heparanase (●) or 20 μl (0.5 μg) of partially purified recombinant heparanase expressed in insect cells (displayed as a bar). Reaction products were analyzed using carbazole assay. Results are expressed in ng glocoronolactone equivalent units per minute (Figure 9a). Heparin sepharose was incubated (37 °C , 17 hours, pH 5.4) with increasing amounts of partially purified recombinant heparanase expressed in insect cells (0.5-1.5 μg) (●) or an enzyme secreted from transformed yeast cells

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(displayed as a bar). Reaction products were analyzed using the dimethylmethylene blue assay (Figure 9b). Results are expressed in ng heparin per minute.

FIG. 10 demonstrates heparanase catalytic activity as a function of the amount of enzyme, using the colorimetric tetrazolium blue assay. Heparan sulfate was incubated (37 °C, 6 hours, pH 5.4) with increasing amounts (0.25-4 µg) of a partially purified heparanase expressed in insect cells. Products of the enzymatic activity were quantified using the tetrazolium blue assay. The results are expressed in ng glucose equivalent units per minute. Each data point represents an average of three independent reactions.

FIG. 11 demonstrates heparanase catalytic activity as a function of incubation time, using the tetrazolium blue assay. Heparan sulfate was incubated (37 °C, pH 5.4) with 3 µg partially purified recombinant heparanase expressed in insect cells for increasing incubation periods (2, 4, 6. 24 hours.). Products of the enzymatic activity were quantified using the tetrazolium blue assay. The results are expressed in µg glucose equivalent (GE) per mg protein. Each data point represents an average of three independent reactions.

FIG. 12 demonstrate screening different potential heparanase inhibitors using the tetrazolium blue assay. Heparan sulfate was incubated (37 °C, 17h, pH 5.4) with 3 µg recombinant heparanase in the absence (-inhibitor) or presence of 10 µg/ml potential inhibitor (as indicated in the Figure). The reaction products were analyzed using the tetrazolium blue assay. The activity was calculated as $\Delta O.D$ of the reaction including the substrate heparan sulfate, the enzyme and the potential inhibitor minus the same reaction but lacking the substrate. The O.D. contributed by the substrate heparan sulfate was also subtracted. The results are expressed as specific activity in glucose equivalent units per milligram protein. Each data point represents an average of three independent reactions.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of a method of screening for heparanase inhibitors which can be used for identifying potential anti-metastatic and anti-inflammatory agents. Specifically. the present invention provides novel qualitative and quantitative colorimetric assays for assaying heparanase catalytic activity.

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The principles and operation of the method according to the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Several studies have shown that heparanase catalytic activity expressed by either normal or neoplastic cells can be effectively inhibited by heparin, modified non-anticoagulant species of heparin and other sulfated polysaccharides (e.g., pentosan polysulfate) and polyanionic molecules (oligonucleotides) (8. 9, 13. 23). Moreover, there was a reasonable good correlation between the heparanase inhibiting activity of these compounds and their ability to inhibit tumor metastasis and inflammatory diseases in experimental animals (8, 13, 17).

The inhibitory effect of heparin on the heparanase enzyme activity is caused presumably due to its being an alternative substrate of the enzyme, as shown in Figure 1. The presence of an alternative substrate which may be more accessible to the enzyme could decrease the catabolism of the ECM by heparanase.

Although many of the inhibitory compounds were anticoagulants, the anti-metastatic and anti-inflammatory potential at the molecules did not correlate with their anti-coagulant activity (8. 13, 17. 32). Moreover, inhibitory polysaccharides did not affect adhesion of tumor cells to the vascular endothelium (33). Parish et al. (13) have demonstrated that sulfated polysaccharides inhibit metastatic dissemination of rat mammary adenocarcinoma cells by inhibiting tumor-cell-derived heparanases involved in the penetration of the vascular endothelium and its underlying basement membrane by tumor cells (13). It is therefore highly important to develop appropriate tools to screen for heparanase inhibiting molecules.

The results described herein in the Examples section emphasize the feasibility of using a both crude and purified human recombinant heparanase to screen. using simple colorimetric assays, polyanionic molecules, chemically modified species of heparin size homogeneous

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oligosaccharides derived from depolymerized heparin. libraries of small molecules and rationally designed molecules for anti-heparanase catalytic activity.

Compounds that inhibited heparanase mediated degradation of heparan sulfate in intact ECM were also found to be potent inhibitors of B16 melanoma and breast carcinoma lung colonization (8), see Table 1 below. These compounds also inhibited several inflammatory diseases (7, 17), pointing toward the clinical significance of the screening method described herein.

Table 1

Compound	Inhibition heparanase	lung colonization
heparin fragment	+	+
N-hexanoyl heparin	+	+
heparin-tetrasaccharide	-	-
heparin tetradeca-saccharide	+	+
totally desulfated heparin	-	-

Effect of heparin species on lung colonization of B16-BL6 melanoma cells. C57BL mice received a single subcutaneous injection of 400 μg/ml of heparin fragment, N-hexanoyl heparin fragment, heparin derived tetrasaccharide, heparin derived tetradeca-saccharide, or totally desulfated fragmin, followed by an intravenous inoculation of B16-BL6 cells (10^5 cells/mouse). Fifteen days afterwards the mice were sacrificed and the lungs fixed in Bouen's solution.

We have demonstrated herein that inhibition of heparanase and tumor metastasis was achieved by heparin species containing 14 sugar units or more and having sulfate groups at both the N and O positions (8). Low sulfate oligosaccharides were less effective heparanase inhibitors than medium and high sulfate fractions of the same size. While O-desulfation abolished the heparanase inhibiting effect of heparin. N-acetylated or N-hexonyl heparin retained a high inhibitory activity, provided that the N-substituted molecules had a molecular size of about 4,000 Dalton or more (8).

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We have previously demonstrated that the presence of N-sulfates is a critical requirement for release of ECM-bound βFGF by species of heparin (20). It was found that total substitution of N-sulfates with acetyl or hexanoyl groups resulted in an almost complete inhibition of the βFGF-releasing activity of heparin, despite a normal content of O-sulfate groups (20). Unlike the inhibition of heparanase catalytic activity and lung colonization, a nearly maximal release of ECM-bound βFGF was obtained already by the octasaccharide, while the tetrasaccharide exhibited about 40 % of the activity of intact heparin when compared on a weight basis (20). No correlation was found between the antithrombotic activity (anti-factor Xa activity) of heparin and its anti-metastatic and βFGF-releasing activities, indicating that the specific pentasaccharide sequence responsible for the binding of anticoagulant heparin to antithrombin III is not required for inhibition of heparanase and release of ECM-bound βFGF.

Heparin or heparan sulfate proteoglycans are involved in receptor binding and mitogenic activity of βFGF (34, 35). We have previously investigated the capacity of various species of heparin and HS to promote β FGF receptor binding using both CHO mutant cells deficient in cell surface HSPG and a soluble βFGF receptor-alkaline phosphatase fusion protein (36). There was an absolute requirement for O-sulfation and a synergistic effect of N-linked sulfates. These structural characteristics of heparin are distinct from those sufficient for displacement of βFGF bound to HS on cell surfaces and ECM (20).

Altogether, these results indicate that different effects of heparin are mediated by different sugar sequences and that unique heparin-like molecules can be designed to elicit or inhibit a specific function. For example. N-substituted species of heparin and in particular N-hexanoyl heparin fragment, rather than native heparin. could be applied to inhibit tumor metastasis, since their efficient inhibition of heparanase catalytic activity was not associated with a significant release of active βFGF from cells and ECM.

These compounds are therefore expected to inhibit metastases formation and inflammatory diseases. correlated with their inhibition of heparanase catalytic activity, with little or no potential induction of neovascularization in response to βFGF release. Similarly, our synthetic polyanionic compounds may exhibit differential activities, provided that appropriate screening systems will be applied. As demonstrated herein, the availability of a recombinant heparanase enzyme and unique heparanase

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assay systems, can be used to identify specific heparanase inhibiting agents. The most potent compounds may then be tested for clinical efficacy *in vivo*.

.   Assays utilizing a radiolabeled substrate can not differentiate between an inhibitory effect of a compound and its being an alternative substrate. In these assays only radiolabeled products are detected, while non-labeled products of an alternative substrate can not be detected. We have modified a simple and convenient semi-quantitative gel analysis (48) in which the substrate as well as its degradation products mediated through heparanase catalytic activity may be visualized on a gel. This method may be used to qualitatively identify heparanase catalytic activity, and also as a simple tool for examining substrate utilization by the enzyme.

Another method presented is the use of an immobilized substrate (heparin-sepharose) and detection of the soluble reaction products by either the colorimetric carbazole or dimethylmethylene blue assays. Similarly, the substrate may be immobilized to microplates such as carbo or hydrazido plates (49). The advantages of these methods are that the activity may be determined using any source of enzyme and not specifically a purified enzyme. Furthermore, these methods do not involve the use of radiolabeled substrates and they obviate the need for size separation. However, these methods are qualitative in nature because. in the carbazole assay, for example, the detection is of uronic (e.g.. iduronic) acids present in a complete hydrolyzate of the products released by heparanase acting on the immobilized substrate. The dimethylmethylene blue assay is similarly qualitative because it detects sulfated glycosaminoglycans released from the immobilized substrate, regardless of their size.

Previous assays developed for the detection of heparanase catalytic activity utilized radiolabeling of the substrate. All these assays are not quantitative, not accurate, they are all time consuming and are not suitable for rapid screening of large number of inhibitors. Here we further describe an assay based on a the detection of newly made reducing ends produced by each cleavage action of the enzyme.

This assay has several advantages over the existing assays and also over the qualitative carbazole and dimethylmethylene blue assays:

First, it allows accurate quantification of heparanase catalytic activity due to detection of any newly formed reducing ends produced by each cleavage action of the enzyme.

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Second, the method is rapid, is not time consuming, simple and does not involve time consuming and difficult preparations of the substrate or separation of the reaction products from the substrate.

Third, the substrate is soluble and therefore is accessible to the enzyme.

Fourth, The substrate is used without undergoing any modifications which may mask or create new cleavage sites for heparanase.

Fifth, the method does not involve the use of radioactive or hazardous materials.

Sixth, the method allows screening of a large number of samples simultaneously.

Seventh, the method utilizes conventional laboratory equipment (e.g., 96 well microplates, microplate reader).

Eighth, the method distinguishes between inhibitory activity of a given compound and its being an alternative substrate.

All these advantages make this assay highly suitable for screening potential anti-metastatic and anti-inflammatory inhibitors using the recombinant mammalian heparanase.

To increase the sensitivity of the assay, the newly formed reducing ends produced by the heparanase may be detected using a fluorigenic reaction with a compound which gives a shift in fluorescence upon reduction (47), such as benzamidine (47) or ANTS (44, 45, 46).

This method in which newly formed reducing ends are detected in microplates may be extended to detect the activity of any enzyme which produces reducing ends, such as glucoronidase, chondroitinase, hyaloronidase, neuraminidase, galactosidase, etc., provided a purified enzyme and substrate are used.

Thus in accordance with one aspect of the teachings of the present invention there is provided a method of testing an agent for its potential at inhibiting heparanase catalytic activity. The method is effected by implementing the following method steps, in which, in a first step a recombinant heparanase enzyme is interacted with a heparanase substrate in the presence of the agent, whereas in a subsequent step of the method the effect of the agent on the catalytic activity of the recombinant heparanase enzyme toward the heparanase substrate is evaluated.

According to another aspect of the present invention there is provided a method of screening for agents inhibiting heparanase catalytic activity and hence potentially inhibiting tumor metastasis, autoimmunity

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and inflammatory diseases. The method is effected by implementing the following method steps, in which in a first step, a recombinant heparanase enzyme is interacted, in individual reactions, with a heparanase substrate in a presence of each of the agents, whereas in a subsequent step of the method the effect of each of the agents tested on the catalytic activity of the recombinant heparanase enzyme toward the heparanase substrate is evaluated.

According to yet another aspect of the present invention there is provided a quantitative method of testing an agent for its potential at inhibiting glycosidase catalytic activity. The method is effected by implementing the following method steps, in which in a first step a glycosidase enzyme is interacted with a glycosidase substrate in a presence of the agent, whereas in a subsequent step of the method the effect of the agent on the catalytic activity of the glycosidase enzyme toward the glycosidase substrate is quantitatively evaluated.

According to still another aspect of the present invention there is provided a method of screening for agents inhibiting heparanase catalytic activity and hence potentially inhibiting tumor metastasis, autoimmunity and inflammatory diseases. The method is effected by implementing the following method steps, in which in a first step a heparanase enzyme is interacted, in individual reactions, with a heparanase substrate in a presence of each of the agents, whereas in a subsequent step of the method the effect of each of the agents on the catalytic activity of the heparanase enzyme toward the heparanase substrate is quantitatively evaluated.

According to an additional aspect of the present invention there is provided a quantitative method of testing glycosidase catalytic activity. The method the method is effected by implementing the following method steps, in which in a first step a glycosidase enzyme is interacted with a glycosidase substrate, whereas in a subsequent step of the method the catalytic activity of the glycosidase enzyme toward the glycosidase substrate is quantitatively evaluated.

As used herein in the specification. the term "qualitative" may also refer to semi-quantitative.

As used herein in the specification and in the claims section below, the phrase "glycosidase catalytic activity" refers to an animal endoglycosidase hydrolyzing activity.

As used herein in the specification and in the claims section below, the phrase "glycosidase enzyme" refers to an enzyme having glycosidase

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catalytic activity. Examples include. but are not limited to, heparanase and types of glucoronidases, chondroitinase. hyaloronidase. neuraminidase, galactosidase, etc.

As used herein in the specification and in the claims section below, the phrase "heparanase catalytic activity" includes animal endoglycosidase hydrolyzing activity which is specific for heparin or heparan sulfate proteoglycan substrates, as opposed to the activity of bacterial enzymes (heparinase I, II and III) which degrade heparin or heparan sulfate by means of β-elimination.

As used herein in the specification and in the claims section below, the phrase "heparanase enzyme" refers to an enzyme having heparanase catalytic activity. Examples include human or any other natural mammalian heparanase which can be purified following the method described in U.S. Pat. No. 5.362.641 to Fuks, which is incorporated by reference as if fully set forth herein. or preferably recombinant mammalian heparanase, genes for which. and the expression and purification of which are described in length in U.S. Pat. application Nos. 08/922.170: 09/071,739; 09/071,618; 09/109,386; 09/258,892; and PCT applications US/17954, US99/09255 and US99/09256, all of which are incorporated herein by reference. It will be appreciated by one ordinarily skilled in the art. and it is demonstrated in the above patent documents, that using the human heparanase gene sequence one can readily clone, express and purify recombinant heparanase of any other mammal. This sequence of events. i.e.. cloning a gene of one species based on the sequence of the same gene from another species, proved successful in hundreds of previous cases. especially since the polymerase chain reaction (PCR) is practiced therefor.

As used herein in the specification and in the claims section below, the phrase "recombinant heparanase enzyme" includes enzymes whose coding sequence have been or will be cloned and are expressed in an expression system. It includes enzymes treated for increased activity. Such treatment can include protease cleavage. It further includes modified sequences including, for example, an introduced protease cleavage site, which when cleaved provides enzyme activation. In particular it includes all the heparanase species described and discussed in U.S. Pat. application No. 09/260,038; and in PCT/US99/09256. which are incorporated by reference as if fully set forth herein.

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As used herein in the specification and in the claims section below, the phrases "heparanase substrate" or "glycosidase substrate" refer to a substrate cleavable into degradation products by the respective enzymes.

As used herein in the specification and in the claims section below, the phrases "inhibiting heparanase catalytic activity" or "inhibiting glycosidase catalytic activity" refers an inhibition of the catalytic activity of the respective enzyme toward a specific substrate in a given assay. Thus, both (other) substrates and inhibitors qualify for inhibiting the catalytic activity of the respective enzymes.

As used herein in the specification and in the claims section below, the phrases "interacted" also means contacted under reaction favorable conditions, i.e., conditions which allow the progress of the reaction to yield reaction products in the absence of inhibition.

The expression system used to express recombinant heparanase according to the present invention may be any suitable expression system. Examples include, but are not limited to, insect cell expression systems, mammalian cell expression systems, and yeast cell expression systems, however, bacterial expression systems are not excluded, see U.S. Pat. application No. 09/071,618. Both crude or purified recombinant heparanase produced in, for example, bacteria, yeast, insect cells or mammalian cells, or any other expression system can be employed in context of the present invention.

The substrate used according to one aspect of the method of the present invention is radiolabeled, using, for example, a radiolabeling method, such as but not limited to *in vitro* radiolabeling and metabolically radiolabeling.

According to one embodiment of the present invention the substrate is an extracellular matrix or a portion thereof. The substrate preferably includes macromolecules associated with the extracellular matrix, such as, but not limited to, heparan sulfate proteoglycans, the natural substrate of various glycosidases including heparanase.

However, according to a presently preferred embodiment of the present invention the substrate is heparan sulfate, heparin, heparin-sepharose, or derivatives thereof.

The agent or agents screened for can be of any type.

One example include anti-heparanase antibodies. It is well known that by binding to the active site antibodies can be used to inhibit catalytic activity of an enzyme.

As used herein in the specification and in the claims section below, the term "antibody" refers to any monoclonal or polyclonal immunoglobulin, or a fragment of an immunoglobin such as sFv (single chain antigen binding protein), Fab1 or Fab2. The immunoglobulin could also be a "humanized" antibody, in which murine variable regions are fused to human constant regions, or in which murine complementarity-determining regions are grafted onto a human antibody structure (Wilder, R.B. et al., J. Clin. Oncol., 14:1383-1400, 1996). Unlike mouse or rabbit antibodies, "humanized" antibodies often do not undergo an undesirable reaction with the immune system of the subject. The terms "sFv" and "single chain antigen binding protein" refer to a type of a fragment of an immunoglobulin, an example of which is sFv CC49 (Larson, S.M. et al., Cancer, 80:2458-68, 1997).

Anti-heparanase antibodies are described in length in U.S. Pat. application No. 09/071,739 and in PCT/US99/09255, which are incorporated by reference as if fully set forth herein. Neutrelizing heparanase antibodies, as described, for example, in U.S. Pat. application No. 09/189,200, which is incorporated herein by reference, are of particular interest for their later use in inactivating heparanase catalytic activity.

Another examples include naturally or man-made (synthetic) agents. Candidate agents for inhibiting heparanase catalytic activity include, but are not limited to, polyanionic molecules, chemically modified species of heparin, size homogeneous oligosaccharides derived from depolymerized heparin, libraries of small molecules (combinatorial library of similar agents) and rationally designed molecules for anti-heparanase catalytic activity. Libraries of synthetic or natural agents are envisaged. Such libraries can be screened for potential hearanase inhibitors in accordance with the teachings of the present invention.

According to one embodiment of the present invention evaluating the catalytic activity of the recombinant heparanase enzyme toward the heparanase substrate and the effect of the agent or agents thereupon is effected by a size separation assay adapted for detection of degradation products of the heparanase substrate. Suitable size separation assays include, bur are not limited to, gel electrophoresis and column chromatography. Other size separation methods are not excluded.

However, according to a presently preferred embodiment of the present invention, quantitatively evaluating the catalytic activity of the glycosidase or heparanase enzyme toward the glycosidase or heparanase

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substrate and the effect of the agent or agents thereupon is effected by an assay adapted for detection of reducing moieties associated with degradation products of the glycosidase or heparanase substrate. The assay is preferably a reducing sugar assay.

Preferably, quantitatively evaluating the catalytic activity of the glycosidase enzyme toward the glycosidase substrate and the effect of the agent or agents thereupon is effected by a colorimetric assay. A suitable colorimetric assay is the tetrazolium blue assay in which tetrazolium blue is reduced to a soluble colored formazan salt. Other quantitative colorimetric assays as, for example, described hereinabove are also envisaged.

As used herein in the specification and in the claims section below, the term "colorimetric" refers to any color producing reaction be it a simple color reaction, which is readily detectable, a fluorimetric reaction or a luminescent (e.g.. chemoluminiscent) reaction, which are readily detectable by fluorescence detecting equipment.

The method preferably behaves substantially linearly in suitable ranges of enzyme concentrations and of time, such that kinetic constants of the assayed enzyme, e.g., under or without inhibition, are readily extractable.

The present invention provides novel qualitative and quantitative assays for determining glycosidase (e.g.. heparanase) catalytic activity, which may therefore be used for screening potential inhibitors of glycosidases, which may serve for therapeutical purposes.

The assays are colorimetric and therefore obviate the need for size separation and for the use of radiolabeled substrates.

The qualitative assays, e.g., the carbazole assay which detects uronic acid derivatives present in complete hydrolyzates of products released from an immobilized substrate, and the dimethylmethylene blue assay, which yields color shift in the presence of polyanionic compounds, are applicable for both crude extracts as well as purified enzymes.

The quantitative assay, on the other hand, is based on the tetrazolium blue assay which colorimetrically detects reducing ends released from the substrate, which may therefore be in either soluble or immobilized form.

The quantitative assay requires pure enzyme and substrate for accuracy and linearity. This however is not a limitation when examining heparanase, for example, which following its cloning and expression is readily available in large amounts.

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As discussed in the Background section above heparanase is involved in tumor cell invasion and metastasis as well as in tumor angiogenesis and it is expressed by cells of the immune system. Therefore, a reliable *in vitro* screening system for heparanase inhibiting compounds may hence be applied to identify and develop non-toxic, anti-cancer drugs which will prevent cell invasion, metastasis and/or tumor angiogenesis, and anti-inflammatory and anti-autoimmune response drugs for the treatment of, for example, multiple sclerosis and other inflammatory and autoimmune diseases.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Materials and Experimental Methods

Materials: Sodium heparin from porcine intestinal mucosa (PM-heparin) (Mr. 14,000, Anti-FXa 165 IU/mg, sulfur content 12 %) was obtained from Hepar Industries (Franklin. Ohio). A low Mr. fragment (Kabi 2165, Fragmin) of this heparin (Mr. 5,100, Anti-FXa 130 IU/mg, sulfur content 12.4 %) was prepared as sodium salt by nitrous acid depolymerization (18). Heparan sulfate from bovine kidney (Cat. No. H 7640). PEG 300 (Cat. No. P 3140), tetrazolium blue chloride (Cat. No. T 4375) and carbazole (Cat. No. C 5132) were purchased from Sigma. Heparin was purchased from Welding. Heparin-sepharose was purchased from Pharmacia. 1,9-Dimethylmethylene blue was purchased from Aldrich (Cat. No. 34108).

Preparation and characterization of oligosaccharides from nitrous acid depolymerized heparins: heparin fragments consisting of saccharide chains of different sizes were obtained by partial deaminative cleavage of heparin from porcine intestinal mucosa using nitrous acid, as described (18). Sizes ranged from disaccharides to chains with an average length of the starting heparin, i.e., chains of about 40-50 saccharides. Size homogeneous oligosaccharides, similar to the starting heparin in their sulfur/carbon ratio, were prepared also by alkaline treatment of heparin methyl ester (β-elimination) as described elsewhere (19). Similar results were obtained. In both cases, the mixture of heparin fragments was subjected to ion exchange chromatography on DEAE Sepharose using sodium chloride as an eluent. Three fractions were collected: low sulfate fraction. sulfur/carbon ratio (by elemental analysis) 0.14; medium sulfate

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fraction, sulfur/carbon ratio 0.19; and high sulfate fraction, sulfur/carbon ratio 0.21 (19, 20). Each of the low sulfate, medium sulfate, and high sulfate fractions was further separated into size-homogenous, even-numbered oligosaccharides by gel permeation chromatography on Sephadex
5   G-50 superfine (19, 20). This procedure resulted in a set of oligosaccharides of different sizes and degrees of sulfation. The oligosaccharides having a higher sulfur/carbon ratio than heparin were found by ^{1}H-NMR and ^{13}C-NMR spectroscopy to have their additional sulfate groups mainly in the glucosamines as indicated by a larger
10   proportion of N-sulfate groups over N-acetyl groups and of 6-O-sulfate groups over 6-OH groups. The low sulfate-containing oligosaccharides had a considerably reduced sulfate content in their glucosamines as well as in their iduronic acids (19).

Modified heparins: Chemically modified non-anticoagulant species
15   of heparin were prepared from native heparin and heparin fragment (Fragmin, Mr. about 5,100). Briefly, the pyridinium salt of heparin and heparin fragment underwent complete N-desulfation by incubation with dimethyl sulfoxide and methanol (21). Total desulfation of N and O sulfate groups was obtained by exhaustive desulfation with dimethyl sulfoxide
20   containing 10 % methanol and 0.4 % trifluoroacetic acid. The N-desulfated heparin fragment was N-acetylated with acetic anhydride in water at pH 7-8 (20), or N-resulfated with sulfur trioxide trimethylamine complex, as described (20). An O-desulfated, N-acetylated heparin fragment was obtained by O-desulfating an N-acetylated heparin fragment, as described
25   (20, 22). Intact heparin was chemically modified by the same procedures. These modified heparins exhibited < 5 % of the anticoagulant activity of heparin (23). The chemical modifications made in the heparin fragments were assessed by ^{1}H NMR and ^{13}C NMR spectroscopy using a JEOL GX-400 instrument, 400 Mhz for ^{1}H and 100 MHz for ^{13}C, and TSP (2,2,3,3-
30   tetradeuterio-3-trimethylsilylpropionate) as an internal standard (23).

Polyanionic molecules: Compounds RG-13577 and P-97100 (polymers of 4-hydroxyphenoxy acetic acid, Mr. about 5,800) and their sulfated variant AV-1/9-1 were synthesized as described (27). Computer assisted molecular modeling suggests that these compounds form a super-
35   structure that ensures a regular spatial distribution of negative charges, similar to that of heparin.

Cells: Cultures of bovine corneal endothelial cells were established from steer eyes as previously described (24). Stock cultures were

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maintained in DMEM (1 g glucose/liter) supplemented with 10 % newborn calf serum, 5 % fetal calf serum (FCS), 50 U/ml penicillin, and 50 μg/ml streptomycin at 37 °C in 10 % CO_2 humidified incubators. Partially purified brain-derived βFGF (100 ng/ml) was added every other day during
5   the phase of active cell growth (20). Highly metastatic B16-BL6 melanoma cells were kindly provided by Dr. I. J. Fidler (University of Texas System Cancer Center, Houston, Texas, 8). Melanoma cells were maintained in culture in DMEM (4.5 g glucose/liter) supplemented with 10 % FCS, L-glutamine and antibiotics.

10   *Preparation of dishes coated with ECM:*   Bovine corneal endothelial cells were dissociated from stock cultures (second to fifth passage) with trypsin/EDTA solution and plated into 4-well plates at a recombinant initial density of $2x10^5$ cells/ml. Cells were maintained as described above except that 5 % dextran T-40 was included in the growth
15 medium and the cells were maintained without addition of βFGF for 12 days. The subendothelial ECM was exposed by dissolving (5 min. room temperature) the cell layer with PBS containing 0.5 % Triton X-100 and 20 mM NH_4OH, followed by four washes in PBS. The ECM remained intact, free of cellular debris and firmly attached to the entire area of the tissue
20 culture dish (11, 20). For preparation of sulfate-labeled ECM, corneal endothelial cells were plated into 4-well plates and cultured as described above. $Na_2[^{35}S]O_4$ (540-590 mCi/mmol) was added (40 μCi/ml) 2 and 5 days after seeding and the cultures were incubated with the label without medium change (11, 20). Ten to twelve days after seeding, the cell
25 monolayer was dissolved and the ECM exposed, as described above.

        To prepare soluble sulfate labeled proteoglycans (peak 1 material), the ECM was digested with trypsin (25 μg/ml, 6 hours, 37 °C), the digest concentrated by reverse dialysis, applied onto a Sepharose 6B gel filtration column and the high molecular weight material (Kav < 0.2, peak I) was
30 collected (25). More than 80 % of the labeled material was shown to be composed of heparan sulfate proteoglycans (11).

        Degradation of sulfated proteoglycans: Sulfate labeled ECM was incubated (3 hours, 37 °C, 10 % CO_2 incubator) with intact cells, or conditioned medium at pH 6.6 and 6.2, respectively. To evaluate the
35 occurrence of proteoglycan degradation, the incubation medium was collected and applied for gel filtration on Sepharose 6B columns (0.9 x 30 cm). Fractions (0.2 ml) were eluted with PBS at a flow rate of 5 ml/hours and counted for radioactivity using Bio-fluor scintillation fluid. The

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excluded volume (Vo) was marked by blue dextran and the total included volume (Vt) by phenol red. The latter was shown to comigrate with free sulfate (11, 20). Degradation fragments of HS side chains were eluted from Sepharose 6B at 0.5 < Kav < 0.8 (peak II. 11. 20). A nearly intact HSPG
5   released from ECM by trypsin - and, to a lower extent, during incubation with PBS alone - was eluted next to Vo (Kav < 0.2, peak I). Recoveries of labeled material applied on the columns ranged from 85 to 95 % in different experiments. Each experiment was performed at least three times and the variation of elution positions (Kav values) did not exceed +/- 15 %.

10 *Tumor metastasis:*   C57BL mice received a single subcutaneous (except when stated otherwise) injection of heparin (400 µg/0.2 ml/mouse, except when stated otherwise) twenty minutes prior to an intravenous inoculation of B16-BL6 melanoma cells (1×10^5 cells/mouse). Mice were sacrificed 15 days later, the lungs fixed in Bouen's solution and scored for
15 the number of metastatic nodules (8).

Expression of heparanase in yeast cells:

Construction of an expression vector for expression in yeast: Construction of the yeast expression vector was a multiple step process. The plasmid pFAST*hpa*2 (described in U.S. Pat. application No.
20 09/071,618) was digested with the restriction enzymes *Stu*I and *Bfr*I. The large fragment isolated was ligated to a PCR amplification product generated as follows: A   pair   of   primers   MF-533   5'-ACCATATGAAAAAGTTCAAGAACAGC-3' (SEQ ID NO:1), in which nucleotides 9-26 correspond to nucleotides 534-551 in SEQ ID NO:9
25 disclosed in PCT/US98/17954 and an antisense *hpa* primer HPL-M1160 5'-CAGCCACATAAAGCCAGCTGC-3' (SEQ ID NO:2) which corresponds to nucleotides 1160-1140 in SEQ ID NO:9 disclosed in PCT/US98/17954, were used in PCR amplification of pFAST*hpa*2. PCR conditions were: denaturation - 94 °C, 40 seconds; annealing - 50 °C, 80 seconds; and
30 elongation - 72 °C, 180 seconds, total of 30 cycles. The PCR product was digested with *Nde*I, followed by treatment with Klenow fragment to generate blunt ended DNA fragments. The blunt product was further digested with *Bfr*I and approximately 220 bp DNA fragment was isolated by gel electrophoresis and was thereafter ligated to the above mentioned
35 pFAST*hpa*2 *Stu*I-*Bfr*I fragment. The resulting plasmid was designated pFAST*hpa*45. pFAST*hpa*45 was digested with *Eco*RI and *Not*I and the insert was then ligated to the *Eco*RI-*Not*I sites of the expression vector

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pPIC3.5K (Invitrogen). The resulting plasmid was designated pPIC3.5K-IMhpa.

Transformation and screening: The yeast *Pichia pastoris* SMD 1168 (*his3, pep4*, Invitrogen) was used as a host for transformation. Transformation and selection were carried out as described in the *Pichia* expression kit protocol (Invitrogen). The expression vector pPIC3.5K-IMhpa was digested with *Sal*I prior to introduction into the yeast cells.

Multiple copy clones were selected using G-418 (Boeheringer Mannehime) as follows: Following transformation. the top agar layer containing the yeast cells was removed and re-suspended in 10 ml of sterile water. Aliquots were removed and plated on YPD plates (1 % yeast extract, 2 % peptone, 2 % glucose) containing increasing concentrations of G-418 (up to 4 mg/ml). Single colonies were picked and streaked again on YPD plates. G-418 resistance was then further confirmed by streaking again isolates on YPD-G-418 plates.

Expression experiments: Single colonies were inoculated into 5 ml buffered glycerol-complex medium (BMGY, 1 % yeast extract. 2 % peptone. 100 mM potassium phosphate buffer pH 6.0, 1.34 % yeast nitrogen base with ammonium sulfate without amino acids, 4×10^{-5} % biotin and 1 % glycerol) and incubated at 30 °C at 250 revolutions per minute (rpm) agitation for 48 hours. Cells were harvested using clinical centrifuge and re-suspended in 3 ml buffered methanol-complex medium (BMMY, the same as BMGY except that 0.5 % methanol replaces the 1 % glycerol). Cells were then incubated at 30 °C at 250 rpm agitation for 48 hours. Culture supernatant (induction medium) of one of the *Pichia pastoris* clones (isolated from 3 mg/ml G-418 YPD plate) was used for detection of human heparanase catalytic activity secreted from yeast.

Heparanase catalytic activity assays:

Polyacrylamide gel analysis of Heparanase catalytic activity: 6 µg heparin (Welding) or heparan sulfate (Sigma) dissolved in H_2O were incubated for 6-24 hours at 37 °C with recombinant purified Heparanase (1 µg) in a 100 µl reaction mixture-buffer A containing 20 mM Phosphate citrate buffer pH 5.4, 1 mM $CaCl_2$, 1 mM NaCl and 1 mM DTT. At the end of incubation period, 0.25 volume of 5x glycerol loading buffer (80 % glycerol. 5 mM CDTA) was added. The samples (50 µl) were fractionated on a 7.5 % (for heparan sulfate samples) or 10 % (for heparin samples) polyacrylamide mini-gel in TAC buffer (40 mM Tris. 20 mM sodium acetate. 1 mM CDTA, pH 7.8). The gel was run for 45 minutes at 100 volts

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and stained with 0.1 % methylene blue in 50 % ethanol for 10 minutes. Destaining was performed with H_2O. Activity was detected as a shift in the mobility of the products produced due to the enzymatic activity as compared to a non treated substrate.

5   *Immobilized substrate:* 100 µl heparin sepharose (50 % suspension in 1 x buffer A) were incubated in 0.5 ml eppendorf tubes placed on a head-over-tail shaker (37 °C, 17 hours) with recombinant heparanase preparations in reaction mixtures containing 20 mM phosphate citrate buffer pH 5.4, 1 mM $CaCl_2$, 1 mM NaCl and 1 mM DTT, in a final volume of 200

10   µl. Enzyme preparations used were either purified recombinant heparanase expressed in insect cells, crude extracts of human 293 cells expressing recombinant human heparanase, or yeast growth medium to which heparanase was secreted from cells expressing recombinant human heparanase. At the end of the incubation time, the samples were centrifuged

15   for 5 minutes at 13,200 rpm. Following incubation, the products released to the supernatant due to the heparanase activity were analyzed using either one of the following calorimetric assays: the carbazole assay or the dimethylmethylene blue assay, as follows.

        Carbazole assay: Supernatants (83 µl) were transferred into glass

20   tubes, 420 µl sulfuric acid tetraborate (2.39 grams sodium tetraborate decahydrate in 250 ml concentrated H_2SO_4) were added, and the samples were boiled for 20 minutes. After boiling , the samples were cooled for 5 minutes on ice and 16.7 µl carbazole reagent (125 grams carbazole in 100 ml ethanol) were added to each sample. The samples were boiled for

25   another 10 minutes. After cooling, the absorbance of the samples was determined using a spectrophotometer (CECIL CE2040) at 530 nm. To each sample a control containing a boiled enzyme was included. In each assay a standard curve containing 3-17 µg glucuronolactone was included. Heparanase activity is expressed as the amount of glucoronolactone

30   equivalent (µg) formed per minute.

        Dimethylmethylene blue assay: Supernatants (100 µl) were transferred to plastic cuvettes. The samples were diluted to 0.5 ml with PBS plus 1 % BSA. 0.5 ml 1,9-Dimethylmethylene blue (32 mg dissolved in 5 ml ethanol and diluted to 1 liter with formate buffer) was added to each

35   sample. Absorbance of the samples was determined using a spectrophotometer (CECIL CE2040) at 530 nm. To each sample a control containing a boiled enzyme was included. In each assay a standard curve containing 1-12 µg low molecular weight heparin   was included.

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Heparanase activity is expressed as the amount of heparin (μg) formed per minute.

Heparanase catalytic activity high-through-put assay: Human recombinant heparanase expressed in insect cells was purified according to as described in U.S. Pat. application Nos. 09/071,739 and 09/071,618, except that DTT was not added to the elution buffer (DTT caused a high background in the Tetrazolium blue assay due to its reducing activity). Heparanase catalytic activity was determined in reactions containing 20 mM Phosphate citrate buffer pH 5.4, 1 mM $CaCl_2$, 1 mM NaCl, 10 % PEG 300 and 50 μg heparan sulfate in a final volume of 100 μl. The reaction was performed in a 96 well microplate with 0.25-4 μg partially purified recombinant heparanase enzyme. The samples were incubated in a 37 °C incubator for 2-24 hours, on a vortex shaker. At the end of incubation, reactions were stopped by adding 100 μl Tetrazolium blue reagent (0.11 % tetrazolium blue in 0.1 M NaOH) to each well. Color was developed by incubation of the plates at 60 °C for 40 minutes. Color intensity was quantitatively determined in a microplate reader (Dynatech) at 580 nm. For each assay a control reaction , not containing the substrate (heparan sulfate) was included. A glucose standard curve of 1-15 μg glucose was also included for each assay. Heparanase catalytic activity was calculated as ΔO.D of the sample containing the substrate minus the sample not containing the substrate. The background O.D. produced by the substrate was also subtracted from all the samples. The results were converted to μg glucose equivalent. One glucose unit is defined as μg glucose equivalent produced per minute. Specific activity is defined as units per mg protein.

Experimental Results

Identifying substrates for heparanase enzyme: Assessment of substrate specificity of the recombinant baculovirus derived human recombinant heparanase toward heparin and heparan sulfate was determined using a modified semi-quantitative polyacrylamide gel assay (48). Using this analysis the substrate (heparin or heparan sulfate) and the heparanase mediated degradation products are separated in a polyacrylamide gel. The gel is stained using methylene blue which reacts with the non treated substrate as well as with the heparin and heparan sulfate fragments. A substrate incubated in the absence of enzyme (control) migrates slower than the digested heparin or heparan sulfate. As shown in Figure 1, with both heparin (lanes a and b) and heparan sulfate (lanes c and d), the degradation products migrate toward the bottom of the gel, indicating that both heparan

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sulfate and heparin are substrates for the human recombinant heparanase enzyme.

Effect of heparin, heparin fragment and oligosaccharides derived from depolymerized heparin on heparanase catalytic activity:

(i) *intact heparin:* Sulfate labeled ECM was incubated (24 hours, 37 °C) with recombinant heparanase in the absence and presence of intact heparin, an alternative substrate of the heparanase enzyme (Figure 2a, 23). Sulfate labeled material released into the incubation medium was analyzed by gel filtration on Sepharose 6B. In the presence of the incubation medium alone, there was a constant release of labeled material that consisted almost entirely (> 90 %) of large Mr. fragments eluted with or next to Vo. We have previously shown that a proteolytic activity residing in the ECM itself (26) is responsible for release of the high Mr material. This nearly intact heparan sulfate proteoglycan provides a soluble substrate for subsequent degradation by heparanase, as also indicated by the relatively large amount of peak-I material accumulating when the heparanase enzyme is apparently inhibited by heparin (Figure, 2a). On the other hand, incubation of the labeled ECM with the recombinant heparanase resulted in release of 60-70 % of the ECM-associated radioactivity in the form of low Mr. sulfate-labeled fragments (peak II, 0.5 < Kav < 0.75. Figure 2a).

In a similar experiment, soluble sulfate labeled peak 1 material (nearly intact ECM-derived heparan sulfate proteoglycans) was incubated with recombinant heparanase in the absence and presence of heparin (Figure 2b). We refer below to heparin and other compounds as heparanase inhibitors, although some or all of which, such as heparin, may be heparanase substrates. Conversion of the radiolabeled high molecular weight peak I substrate into low molecular weight peak II material was completely inhibited, in the presence of 1 μg/ml heparin, (Figure 2b). This effect is probably due to the excess of the non-labeled substrate (heparin) since the addition of substrate in excess may interfere with heparanase endogenic substrates. Degradation fragments eluted in peak II were shown to be degradation products of heparan sulfate, as they were (i) 5 to 6 fold smaller than intact heparan sulfate side chains (Kav about 0.33) released from ECM by treatment with either alkaline borohydride or papain; and (ii) resistant to further digestion with papain or chondroitinase ABC (not shown) and susceptible to deamination by nitrous acid (Figure 3).

(ii) *heparin-derived oligosaccharides:* We investigated the inhibitory effect of size homogenous oligosaccharides containing 4 and 14

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sugar units, prepared from heparin by nitrous acid depolymerization (Figure 4). As demonstrated in Figure 4, complete inhibition of recombinant heparanase was observed in the presence of 1 µg/ml of the tetradeca-saccharide (91420-14 units). A similar result was observed with heparin derived oligosaccharides containing 16 and 18 sugar units (not shown), while there was a very slight inhibition by 1 µg/ml of the tetrasaccharide (90420-4 units). Similar results were obtained with oligosaccharides derived from heparin by nitrous acid depolymerization (Figure 4. 84588-Fragmin) or by alkaline β-elimination (not shown).

(iii) Chemically modified species of heparin: Chemically modified non-anticoagulant species of heparin fragment (low molecular weight heparin. Fragmin, Mr. of about 5,100.). prepared as described under Experimental Methods, were incubated with sulfate labeled ECM in the absence and presence of heparin fragment (84588-Fragmin). N/O totally desulfated heparin fragment (sulfate < 1 %. 70334-N/O desulfated), and either N-acetylated (70421-tot) or N-hexanoylated (70901-tot) heparin fragments. Accumulation in the medium of low Mr. sulfate labeled degradation fragments was completely inhibited in the presence of 1 µg/ml heparin fragment, but there was only a slight inhibition by 10 µg/ml N/O desulfated heparin fragment (Figure 5). There was no inhibition of heparanase catalytic activity by 1 µg/ml of N-desulfated (sulfate equals 9.7 %), or N/O-desulfated, N-resulfated (sulfate equals 5.3 %) species of heparin (not shown), indicating that both the N- and O-sulfates of heparin are required for inhibition of recombinant heparanase. Substitution of the N-sulfates of heparin fragment with acetyl or hexanoyl groups (sulfate equals 8.7 % and 7.5 %, respectively) yielded species of heparin which inhibited heparanase catalytic activity nearly as well as heparin fragment (Figure 5; i.e., complete inhibition in the presence of 1 µg/ml).

Our screening experiments using the recombinant heparanase enzyme indicated that while O-desulfation abolished the heparanase inhibiting effect of heparin, O-sulfated and N-substituted (e.g., N-acetyl or N-hexanoyl) species of heparin retained a high inhibitory activity. Inhibition of recombinant heparanase was best achieved by heparin species containing 16 sugar units or more and having sulfate groups at both the N and O positions. Low sulfate oligosaccharides were less effective heparanase inhibitors than medium and high sulfate fractions of the same size saccharide. Heparin fractions with high and low affinity to anti-

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thrombin III exhibited a similar high heparanase inhibitory activity. despite a 200 fold difference in their anti-coagulant activity (not shown).

(iv) Synthetic polyanionic heparin-mimicking compounds: heparin and heparan sulfate (HS) participate in the regulation of many cellular processes, particularly as they relate to cell growth and differentiation, tumor metastasis and angiogenesis, autoimmunity, lipoprotein metabolism, and gene expression, although its mode of action has not been clearly elucidated (2). Compounds that modulate functional properties of heparin and/or HS are therefore of increasing importance for the development of therapeutic agents of widespread utility. In collaboration with Rhone-Poulenc Rorer Co., we have synthesized potent mimetics of heparin/HS and investigated their mode of action and possible application in the inhibition of restenosis, angiogenesis and tumor progression (27-31). For this purpose. we applied the acid-catalyzed polymerization of phenols and formaldehyde to synthesize negatively charged, non-sulfated polyanionic compounds (27, U.S. Pat. No. 5,674,482, which is incorporated by reference as if fully set forth herein). Aromatic ring monomers were chosen and strong oxidizing reagents avoided so that the polymerization process with formaldehyde would yield substantially ordered and defined backbones. Using this approach, a series of non-toxic linear polyanionic polymers was identified with repeating phenol-based monomers, including poly-4-hydroxyphenoxy acetic acid, hereby termed compound RG-13577, that mimics some of the effects of heparin. Of particular significance is the ability of these compounds to inhibit cell proliferation (e.g., vascular endothelial and smooth muscle cells, mesangial cells, 28-30), and revert the transformed phenotype of basic fibroblast growth factor (βFGF) transfected cells (31).

With respect to the detailed chemical structure. there are several differences between RG-13577 and heparin. While heparin contains sulfonate as a major anionic group, RG-13577 contains only carboxylic residues. In addition, RG-13577 is composed of aromatic rings linked by an enzyme resistant methylene group, while heparin has a biodegradable sugar backbone. RG-13577 and heparin represent, each, a family of polymers of repeating units. The estimated molecular weight of the active compound of RG-13577 is around 8,000, while the molecular weight of commercially available heparin is within the range of 5,000-30,000. Recently, two polyanionic compounds were synthesized by InSight Ltd., Israel. One, termed P-97100. is identical to compound RG-13577 and the second is a

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sulfated version of compound P-97100, in which the carboxyl groups are replaced with sulfated groups. Using the recombinant heparanase enzyme and the above described assay systems. we have screened the ability of these compounds to inhibit heparanase catalytic activity.

Soluble, sulfate-labeled ECM-derived HSPG substrate (peak I material) was incubated with purified recombinant heparanase in the absence and presence of increasing concentrations (1, 5 and 10 µg/ml) of heparin (Figure 6a), compound P-97100 (poly-4-hydroxyphenoxy acetic acid. synthesized by InSight, Figure 6b), or compound RG-13577 (poly-4-hydroxyphenoxy acetic acid, synthesized by RhOne-Poulenc Rorer, Figure 6c). Following 18 hours incubation (37 °C, pH 5.8). the reaction mixtures were subjected to gel filtration over Sepharose 6B and analyzed for conversion of the peak I substrate into low molecular weight degradation fragments. Complete inhibition of this conversion (heparanase catalytic activity) was obtained in the presence of 5 µg/ml heparin (Figure 6a) or 10 µg/ml complete inhibition of this conversion (heparanase catalytic activity) was obtained in the presence of 5 µg/ml heparin or 10 µg/ml of each of the other compounds (Figures 6b-c). Heparin and compound P-97100 exerted a small inhibitory effect already at 1 µg/ml (Figures 6a-b). while there was no effect to RG-13577 at this concentration (Figure 6c). It appears that in this assay system, compound P-97100 prepared by InSight was slightly more effective than RG-13577 and nearly as active as heparin.

Intact, sulfate labeled ECM was applied to compare the heparanase inhibiting activity of compound AV-1/9-1 versus compound P-97100. As demonstrated in Figure 7a, complete inhibition of recombinant heparanase catalytic activity was obtained in the presence of 0.5 and 1 µg/ml compound P-97100. An almost complete inhibition was obtained at 0.1 µg/ml compound P-97100. Under the same conditions, compound AV-1/9-1 (sulfated poly-4-hydroxyphenoxy acetic acid, synthesized by InSight) yielded a partial inhibition at 0.5 and 1 µg/ml, but there was no inhibition at 0.1 µg/ml AV-1/9-1 (Figure 7b). These results indicate that compound P-97100 is a more potent inhibitor of heparanase catalytic activity as compared to its sulfated analog AV-1/9-1 and that the recombinant heparanase enzyme can be applied for screening purposes and detection of even small differences between potential heparanase inhibiting compounds.

Heparanase catalytic activity using an immobilized substrate: To determine whether the recombinant heparanase could utilize an immobilized substrate. a reaction was performed with heparin-sepharose as a substrate

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and the products of the reaction were observed using a polyacrylamide gel assay. As shown in Figure 8. a shift in the mobility of the products of the enzymatic reaction can be detected, indicating that the recombinant heparanase can cleave an immobilized substrate.

In an attempt to develop an assay which may quantify the reaction products using crude or purified heparanase. a similar incubation of the enzyme with heparin-sepharose was made. but the products were analyzed using a colorimetric method, either the carbazole assay, which allows estimation of uronic acids, such as iduronic acids, of heparin and heparan sulfate or lactone forms of uronic acid, such as glucoronolactone (43). or the dimethylmethylene blue assay, which allows estimation of sulfated glycosaminoglycans (51).

As shown in Figure 9a, recombinant Heparanase catalytic activity expressed in either mammalian or insect cells could be quantitatively detected using the carbazole assay. The activity was found to be about 6 fold higher in the heparanase expressed in mammalian cells, although a crude preparation was used for the mammalian cells whereas the enzyme expressed in insect cells was partially purified. Similarly. as shown in Figure 9b, products of heparanase activity. expressed in either yeast cells or insect cells, could be detected using the dimethylmethylene blue assay (Figure 9b). The activity per μl is higher for the enzyme expressed in insect cells but the amount of heparanase expressed in yeast is much lower. From yeast, a preparation of induction medium (to which heparanase was secreted) was tested, whereas a purified enzyme was used for the enzyme expressed in insect cells.

High throughput heparanase catalytic activity assay for screening for potential anti-metastatic and anti-inflammatory agents using recombinant mammalian heparanase as a probe: Before studying the potential at the novel assay to be used for screening of potential inhibitors, it was necessary to demonstrate that the assay has linear ranges on a soluble substrate with increasing amounts of enzyme and incubation times. Recombinant heparanase, expressed in insect cells and partially purified, was used for determination of activity. PEG 300 was added to the reaction mixture to stabilize the enzyme. Increasing amounts of enzyme, 0.25-4 μg heparanase, were added to the reaction mixture and incubation was made for 6 hours at 37 °C. As shown in Figure 10. the activity of heparanase was linear with increasing amounts of enzyme. from 0.25-4 μg protein.

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Next, the linearity of the activity with respect to time was examined. Reactions were incubated for 2, 4, 6 and 24 hours with 3 μg of enzyme in each reaction. As shown in Figure 11, the activity was linear for up to 24 hours.

In order to determine whether this assay is suitable for screening for heparanase inhibitors, several different species of heparin (chemically modified) and heparin-mimicking compounds were tested for their inhibitory effect on heparanase catalytic activity, using the recombinant enzyme and the new assay system. One or 10 μg/ml of each potential inhibitor was added to a reaction including or lacking the substrate heparan sulfate. Each reaction contained 3 μg of enzyme and was incubated for 17 hours at 37 °C. The reactions in which the potential inhibitor was included were compared to a reaction not containing the compound. The background caused due to the substrate was subtracted from all activity results. The results of the effect of the different potential inhibitors at a concentration of 10 μg/ml are presented in Figure 12. In general, the results of the inhibition of heparanase using this assay correlate well with the results obtained with labeled ECM. Compounds 70421-N-desulfated, N-acetylated Fragmin, 70901-N-desulfated, and N-hexanoylated Fragmin completely inhibited heparanase catalytic activity, while compound 70334-N/O desulfated Fragmin did not cause significant inhibition. The synthetic polyanionic heparin-mimetic, RG-13577 (25) partially inhibited heparanase catalytic activity.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

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SEQUENCE LISTING

(1)   GENERAL INFORMATION:
       (i)   APPLICANT: Hanna Ben-Artzi et al.
       (ii)   TITLE OF INVENTION: METHOD OF SCREENING FOR POTENTIAL ANTI-
                                      METASTATIC AND ANTI-INFLAMMATORY AGENTS
USING

                                      MAMMALIAN HEPARANASE AS A PROBE
       (iii) NUMBER OF SEQUENCES: 2
       (iv)   CORRESPONDENCE ADDRESS:
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              (D)   STATE: Virginia
              (E)   COUNTRY: United States of America
              (F)   ZIP: 22202
       (v)   COMPUTER READABLE FORM:
              (A)   MEDIUM TYPE: 1.44 megabyte, 3.5" microdisk
              (B)   COMPUTER: Twinhead® Slimnote-890TX
              (C)   OPERATING SYSTEM: MS DOS version 6.2.
                                      Windows version 3.11
              (D)   SOFTWARE: Word for Windows version 2.0 converted
to
                                      an ASCI file
       (vi)   CURRENT APPLICATION DATA:
              (A)   APPLICATION NUMBER:
              (B)   FILING DATE:
              (C)   CLASSIFICATION:
       (vii) PRIOR APPLICATION DATA:
              (A)   APPLICATION NUMBER: 09/113,168
              (B)   FILING DATE: July 10, 1998
       (viii) ATTORNEY/AGENT INFORMATION:
              (A)   NAME: Friedmam, Mark M.
              (B)   REGISTRATION NUMBER: 33,883
              (C)   REFERENCE/DOCKET NUMBER: 910/24
       (ix)   TELECOMMUNICATION INFORMATION:
              (A)   TELEPHONE: 972-3-5625553
              (B)   TELEFAX: 972-3-5625554
              (C)   TELEX:
(2)   INFORMATION FOR SEQ ID NO:1:
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              (C)   STRANDEDNESS: single
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WHAT IS CLAIMED IS:

1.   A method of testing an agent for its potential at inhibiting heparanase catalytic activity, the method comprising the steps of interacting a recombinant heparanase enzyme with a heparanase substrate in a presence of the agent and evaluating an effect of the agent on the catalytic activity of said recombinant heparanase enzyme toward said heparanase substrate.

2.   The method of claim 1, wherein said recombinant heparanase is of a human origin.

3.   The method of claim 1, wherein said recombinant heparanase is expressed in an expression system selected from the group consisting of an insect cell expression system, a mammalian cell expression system and a yeast cell expression system.

4.   The method of claim 1, wherein said heparanase substrate is radiolabeled.

5.   The method of claim 1, wherein said radiolabeled heparanase substrate is radiolabeled by a radiolabeling method selected from the group consisting of in vitro radiolabeling and metabolically radiolabeling.

6.   The method of claim 1, wherein said heparanase substrate is an extracellular matrix or a portion thereof.

7.   The method of claim 6, wherein said heparanase substrate includes macromolecules associated with said extracellular matrix.

8.   The method of claim 7, wherein said macromolecules include heparan sulfate proteoglycans.

9.   The method of claim 1, wherein said heparanase substrate includes extracellular matrix-derived soluble heparan sulfate proteoglycans.

10.   The method of claim 1, wherein said heparanase substrate is selected from the group consisting of heparan sulfate, heparin, heparin-sepharose, and derivatives thereof.

11.   The method of claim 1, wherein said heparanase substrate is immobilized.

12.   The method of claim 1, wherein the agent is an anti-heparanase antibody.

13.   The method of claim 1, wherein the agent is a naturally occurring agent.

14.   The method of claim 1, wherein the agent is a synthetic agent or a library of synthetic agents.

15.   The method of claim 14, wherein said synthetic agent is one of a plurality of agents belonging to a combinatorial library of similar agents.

16.   The method of claim 1, wherein evaluating said effect of the agent on the catalytic activity of said recombinant heparanase enzyme toward said heparanase substrate is effected by a size separation assay adapted for detection of degradation products of said heparanase substrate.

17.   The method of claim 16, wherein said size separation assay is selected from the group consisting of gel electrophoresis and column chromatography.

18.   The method of claim 1, wherein evaluating said effect of the agent on the catalytic activity of said recombinant heparanase enzyme toward said heparanase substrate is effected by a colorimetric assay.

19.   The method of claim 18, wherein said colorimetric assay is selected from the group consisting of carbazole assay and methylene blue assay.

20.   A method of screening for agents inhibiting heparanase catalytic activity and hence potentially inhibiting tumor metastasis, autoimmunity and inflammatory diseases, the method comprising the steps of, in individual reactions, interacting a recombinant heparanase enzyme with a heparanase substrate in a presence of each of the agents and

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evaluating an effect of each of the agents on the catalytic activity of said recombinant heparanase enzyme toward said heparanase substrate.

21.   The method of claim 20, wherein said recombinant heparanase is of a human origin.

22.   The method of claim 20, wherein said recombinant heparanase is expressed in an expression system selected from the group consisting of an insect cell expression system, a mammalian cell expression system and a yeast cell expression system.

23.   The method of claim 20, wherein said heparanase substrate is radiolabeled.

24.   The method of claim 20, wherein said radiolabeled heparanase substrate is radiolabeled by a radiolabeling method selected from the group consisting of in vitro radiolabeling and metabolically radiolabeling.

25.   The method of claim 20, wherein said heparanase substrate is an extracellular matrix or a portion thereof.

26.   The method of claim 25, wherein said heparanase substrate includes macromolecules associated with said extracellular matrix.

27.   The method of claim 26, wherein said macromolecules include heparan sulfate proteoglycans.

28.   The method of claim 20, wherein said heparanase substrate includes extracellular matrix-derived soluble heparan sulfate proteoglycans.

29.   The method of claim 20, wherein said heparanase substrate is selected from the group consisting of heparan sulfate, heparin, heparin-sepharose, and derivatives thereof.

30.   The method of claim 20, wherein said heparanase substrate is immobilized.

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31.   The method of claim 20, wherein the agents include at least one anti-heparanase antibody.

32.   The method of claim 20, wherein the agents include at least one naturally occurring agent.

33.   The method of claim 20, wherein the agents include at least one synthetic agent or a library of synthetic agents.

34.   The method of claim 33, wherein said at least one synthetic agent is one of a plurality of agents belonging to a combinatorial library of similar agents.

35.   The method of claim 20, wherein evaluating said effect of each of the agents on the catalytic activity of said recombinant heparanase enzyme toward said heparanase substrate is effected by a size separation assay adapted for detection of degradation products of said heparanase substrate.

36.   The method of claim 35, wherein said size separation assay is selected from the group consisting of gel electrophoresis and column chromatography.

37.   The method of claim 20, wherein evaluating said effect of the agent on the catalytic activity of said recombinant heparanase enzyme toward said heparanase substrate is effected by a colorimetric assay.

38.   The method of claim 37, wherein said colorimetric assay is selected from the group consisting of carbazole assay and dimethylmethylene blue.

39.   A quantitative method of testing an agent for its potential at inhibiting glycosidase catalytic activity, the method comprising the steps of interacting a glycosidase enzyme with a glycosidase substrate in a presence of the agent and quantitatively evaluating an effect of the agent on the catalytic activity of said glycosidase enzyme toward said glycosidase substrate.

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40.   The quantitative method of claim 39. wherein said glycosidase enzyme is a heparanase enzyme and said glycosidase substrate is. respectively, a heparanase substrate.

41.   The quantitative method of claim 40, wherein said heparanase is a natural heparanase.

42.   The quantitative method of claim 40, wherein said heparanase is a recombinant heparanase.

43.   The quantitative method of claim 42. wherein said recombinant heparanase is of a human origin.

44.   The quantitative method of claim 42, wherein said recombinant heparanase is expressed in an expression system selected from the group consisting of an insect cell expression system, a mammalian cell expression system and a yeast cell expression system.

45.   The quantitative method of claim 40, wherein said heparanase substrate is an extracellular matrix or a portion thereof.

46.   The quantitative method of claim 45, wherein said heparanase substrate includes macromolecules associated with said extracellular matrix.

47.   The quantitative method of claim 46, wherein said macromolecules include heparan sulfate proteoglycans.

48.   The quantitative method of claim 40, wherein said heparanase substrate includes extracellular matrix-derived soluble heparan sulfate proteoglycans.

49.   The quantitative method of claim 40, wherein said heparanase substrate is selected from the group consisting of heparan sulfate, heparin, heparin-sepharose, and derivatives thereof.

50.   The quantitative method of claim 40, wherein said heparanase substrate is immobilized.

51.	The quantitative method of claim 40, wherein the agent is an anti-heparanase antibody.

52.	The quantitative method of claim 40, wherein the agent is a naturally occurring agent.

53.	The quantitative method of claim 40, wherein the agent is a synthetic agent.

54.	The quantitative method of claim 53, wherein said synthetic agent is one of a plurality of agents belonging to a combinatorial library of similar agents.

55.	The quantitative method of claim 39, wherein quantitatively evaluating said effect of the agent on the catalytic activity of said glycosidase enzyme toward said glycosidase substrate is effected by an assay adapted for detection of reducing moieties associated with degradation products of said glycosidase substrate.

56.	The quantitative method of claim 55, wherein said glycosidase enzyme is a heparanase enzyme and said glycosidase substrate is, respectively, a heparanase substrate.

57.	The quantitative method of claim 55, wherein said assay is colorimetric.

58.	The quantitative method of claim 55, wherein said assay is a reducing sugar assay.

59.	The quantitative method of claim 57, wherein said colorimetric assay is a tetrazolium blue assay in which tetrazolium blue is reduced to a soluble colored formazan salt.

60.	The quantitative method of claim 39, wherein quantitatively evaluating said effect of the agent on the catalytic activity of said glycosidase enzyme toward said glycosidase substrate is effected by a colorimetric assay.

61. The quantitative method of claim 60. wherein said glycosidase enzyme is a heparanase enzyme and said glycosidase substrate is. respectively, a heparanase substrate.

62. The quantitative method of claim 60. wherein said colorimetric assay is adapted for detection of reducing moieties associated with degradation products of said glycosidase substrate.

63. The quantitative method of claim 62, wherein said assay is a reducing sugar assay.

64. The quantitative method of claim 60, wherein said colorimetric assay is a tetrazolium blue assay in which tetrazolium blue is reduced to a soluble colored formazan salt.

65. The quantitative method of claim 39, wherein said method behaves substantially linearly in a range of enzyme concentration.

66. The quantitative method of claim 39. wherein said method behaves substantially linearly in a range of time.

67. A quantitative method of screening for agents inhibiting heparanase catalytic activity and hence potentially inhibiting tumor metastasis, autoimmunity and inflammatory diseases, the method comprising the steps of, in individual reactions, interacting a heparanase enzyme with a heparanase substrate in a presence of each of the agents and quantitatively evaluating an effect of each of the agents on the catalytic activity of said heparanase enzyme toward said heparanase substrate.

68. The quantitative method of claim 67, wherein said heparanase is a natural heparanase.

69. The quantitative method of claim 67, wherein said heparanase is a recombinant heparanase.

70. The quantitative method of claim 69. wherein said recombinant heparanase is of a human origin.

71.   The quantitative method of claim 69, wherein said recombinant heparanase is expressed in an expression system selected from the group consisting of an insect cell expression system, a mammalian cell expression system and a yeast cell expression system.

72.   The quantitative method of claim 67, wherein said heparanase substrate is an extracellular matrix or a portion thereof.

73.   The quantitative method of claim 72, wherein said heparanase substrate includes macromolecules associated with said extracellular matrix.

74.   The quantitative method of claim 73, wherein said macromolecules include heparan sulfate proteoglycans.

75.   The quantitative method of claim 67, wherein said heparanase substrate includes extracellular matrix-derived soluble heparan sulfate proteoglycans.

76.   The quantitative method of claim 67, wherein said heparanase substrate is selected from the group consisting of heparan sulfate, heparin, heparin-sepharose, and derivatives thereof.

77.   The method of claim 67, wherein said heparanase substrate is immobilized.

78.   The quantitative method of claim 67, wherein the agents include at least one anti-heparanase antibody.

79.   The quantitative method of claim 67, wherein the agents include at least one naturally occurring agent.

80.   The quantitative method of claim 67, wherein the agents include at least one synthetic agent.

81.   The quantitative method of claim 80, wherein said at least one synthetic agent is one of a plurality of agents belonging to a combinatorial library of similar agents.

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82.   The quantitative method of claim 67, wherein quantitatively evaluating said effect of the agent on the catalytic activity of said heparanase enzyme toward said heparanase substrate is effected by an assay adapted for detection of reducing moieties associated with degradation products of said heparanase substrate.

83.   The quantitative method of claim 82, wherein said assay is colorimetric.

84.   The quantitative method of claim 82, wherein said assay is a reducing sugar assay.

85.   The quantitative method of claim 84, wherein said colorimetric assay is a tetrazolium blue assay in which tetrazolium blue is reduced to a soluble colored formazan salt.

86.   The quantitative method of claim 67, wherein quantitatively evaluating said effect of the agent on the catalytic activity of said heparanase enzyme toward said heparanase substrate is effected by a colorimetric assay.

87.   The quantitative method of claim 86, wherein said colorimetric assay is adapted for detection of reducing moieties associated with degradation products of said heparanase substrate.

88.   The quantitative method of claim 87, wherein said assay is a reducing sugar assay.

89.   The quantitative method of claim 86, wherein said colorimetric assay is a tetrazolium blue assay in which tetrazolium blue is reduced to a soluble colored formazan salt.

90.   The quantitative method of claim 67, wherein said method behaves substantially linearly in a range of enzyme concentration.

91.   The quantitative method of claim 67, wherein said method behaves substantially linearly in a range of time.

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92.   A quantitative method of testing glycosidase catalytic activity, the method comprising the steps of interacting a glycosidase enzyme with a glycosidase substrate and quantitatively evaluating the catalytic activity of said glycosidase enzyme toward said glycosidase substrate.

93.   The quantitative method of claim 92, wherein said glycosidase enzyme is a heparanase enzyme and said glycosidase substrate is, respectively, a heparanase substrate.

94.   The quantitative method of claim 93, wherein said heparanase is a natural heparanase.

95.   The quantitative method of claim 93, wherein said heparanase is a recombinant heparanase.

96.   The quantitative method of claim 95, wherein said recombinant heparanase is of a human origin.

97.   The quantitative method of claim 95, wherein said recombinant heparanase is expressed in an expression system selected from the group consisting of an insect cell expression system, a mammalian cell expression system and a yeast cell expression system.

98.   The quantitative method of claim 93, wherein said heparanase substrate is an extracellular matrix or a portion thereof.

99.   The quantitative method of claim 98, wherein said heparanase substrate includes macromolecules associated with said extracellular matrix.

100.   The quantitative method of claim 99, wherein said macromolecules include heparan sulfate proteoglycans.

101.   The quantitative method of claim 93, wherein said heparanase substrate includes extracellular matrix-derived soluble heparan sulfate proteoglycans.

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102.   The quantitative method of claim 93. wherein said heparanase substrate is selected from the group consisting of heparan sulfate. heparin, heparin-sepharose, and derivatives thereof.

103.   The quantitative method of claim 93. wherein said heparanase substrate is immobilized.

104.   The quantitative method of claim 92, wherein quantitatively evaluating the catalytic activity of said glycosidase enzyme toward said glycosidase substrate is effected by an assay adapted for detection of reducing moieties associated with degradation products of said glycosidase substrate.

105.   The quantitative method of claim 104. wherein said glycosidase enzyme is a heparanase enzyme and said glycosidase substrate is. respectively. a heparanase substrate.

106.   The quantitative method of claim 104, wherein said assay is colorimetric.

107.   The quantitative method of claim 104, wherein said assay is a reducing sugar assay.

108.   The quantitative method of claim 106, wherein said colorimetric assay is a tetrazolium blue assay in which tetrazolium blue is reduced to a soluble colored formazan salt.

109.   The quantitative method of claim 92, wherein quantitatively evaluating the catalytic activity of said glycosidase enzyme toward said glycosidase substrate is effected by a colorimetric assay.

110.   The quantitative method of claim 109, wherein said glycosidase enzyme is a heparanase enzyme and said glycosidase substrate is. respectively, a heparanase substrate.

111.   The quantitative method of claim 109, wherein said colorimetric assay is adapted for detection of reducing moieties associated with degradation products of said glycosidase substrate.

112. The quantitative method of claim 111. wherein said assay is a reducing sugar assay.

113. The quantitative method of claim 109. wherein said colorimetric assay is a tetrazolium blue assay in which tetrazolium blue is reduced to a soluble colored formazan salt.

114. The quantitative method of claim 92. wherein said method behaves substantially linearly in a range of enzyme concentration.

115. The quantitative method of claim 92. wherein said method behaves substantially linearly in a range of time.

116. A method of testing heparanase catalytic activity. the method comprising the steps of interacting a recombinant heparanase enzyme with a heparanase substrate and evaluating the catalytic activity of said recombinant heparanase enzyme toward said heparanase substrate.

117. The method of claim 116. wherein said recombinant heparanase is of a human origin.

118. The method of claim 116, wherein said recombinant heparanase is expressed in an expression system selected from the group consisting of an insect cell expression system. a mammalian cell expression system and a yeast cell expression system.

119. The method of claim 116. wherein said heparanase substrate is radiolabeled.

120. The method of claim 116. wherein said radiolabeled heparanase substrate is radiolabeled by a radiolabeling method selected from the group consisting of in vitro radiolabeling and metabolically radiolabeling.

121. The method of claim 116. wherein said heparanase substrate is an extracellular matrix or a portion thereof.

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122.   The method of claim 121. wherein said heparanase substrate includes macromolecules associated with said extracellular matrix.

123.   The method of claim 122. wherein said macromolecules include heparan sulfate proteoglycans.

124.   The method of claim 116. wherein said heparanase substrate includes extracellular matrix-derived soluble heparan sulfate proteoglycans.

125.   The method of claim 116. wherein said heparanase substrate is selected from the group consisting of heparan sulfate, heparin, heparin-sepharose, and derivatives thereof.

126.   The method of claim 116. wherein said heparanase substrate is immobilized.

127.   The method of claim 116. wherein the agent is an anti-heparanase antibody.

128.   The method of claim 116. wherein evaluating said effect of the catalytic activity of said recombinant heparanase enzyme toward said heparanase substrate is effected by a size separation assay adapted for detection of degradation products of said heparanase substrate.

129.   The method of claim 128. wherein said size separation assay is selected from the group consisting of gel electrophoresis and column chromatography.

130.   The method of claim 116. wherein evaluating said effect of the catalytic activity of said recombinant heparanase enzyme toward said heparanase substrate is effected by a colorimetric assay.

131.   The method of claim 130. wherein said colorimetric assay is selected from the group consisting of carbazole assay and methylene blue assay.

Fig. 1

Fig. 2a

Fig. 2b

Fig. 3

Fig. 4

Fig. 5

4/8
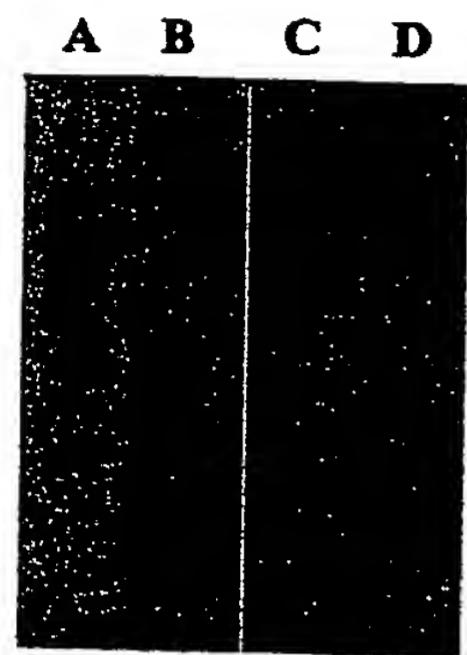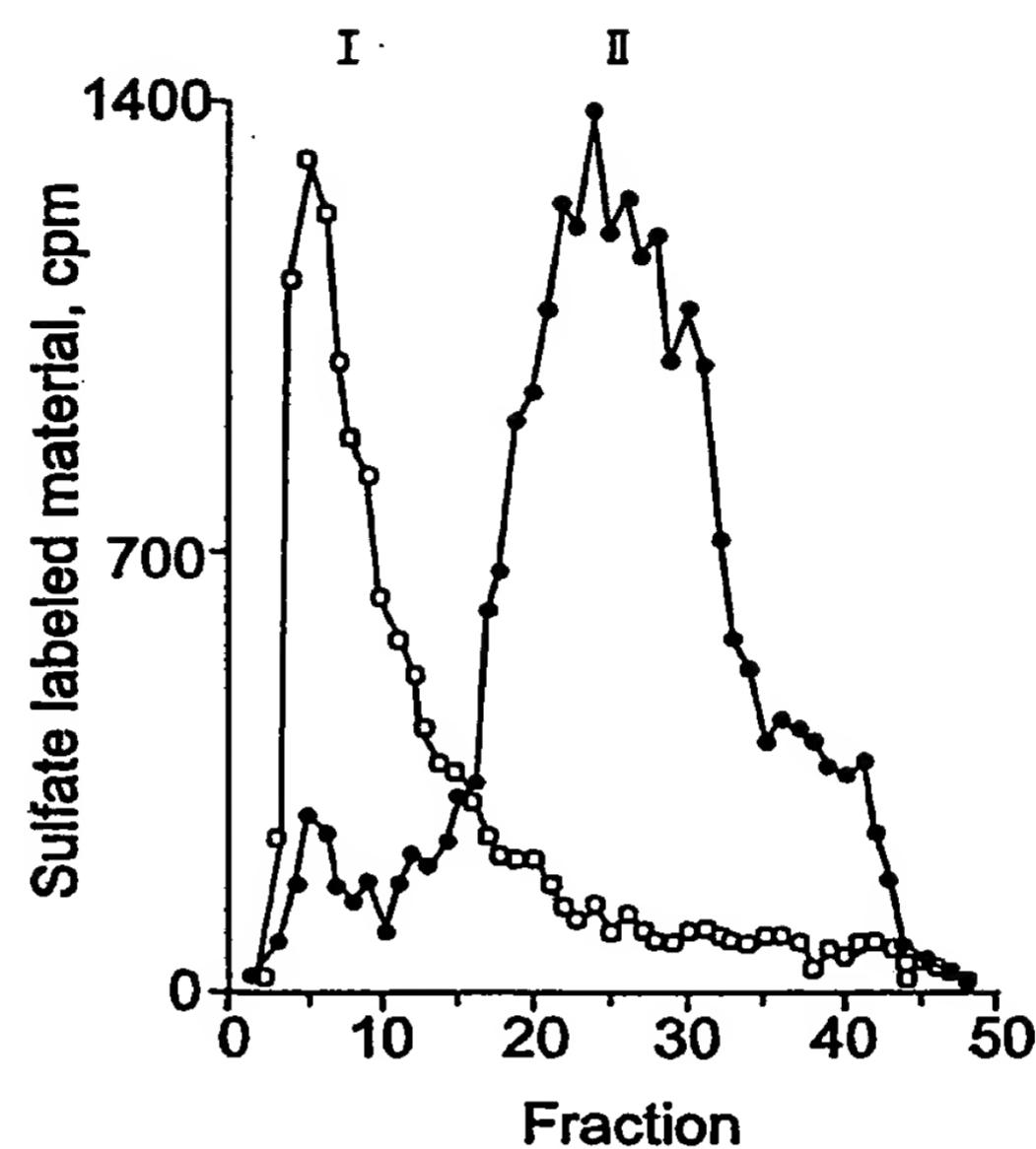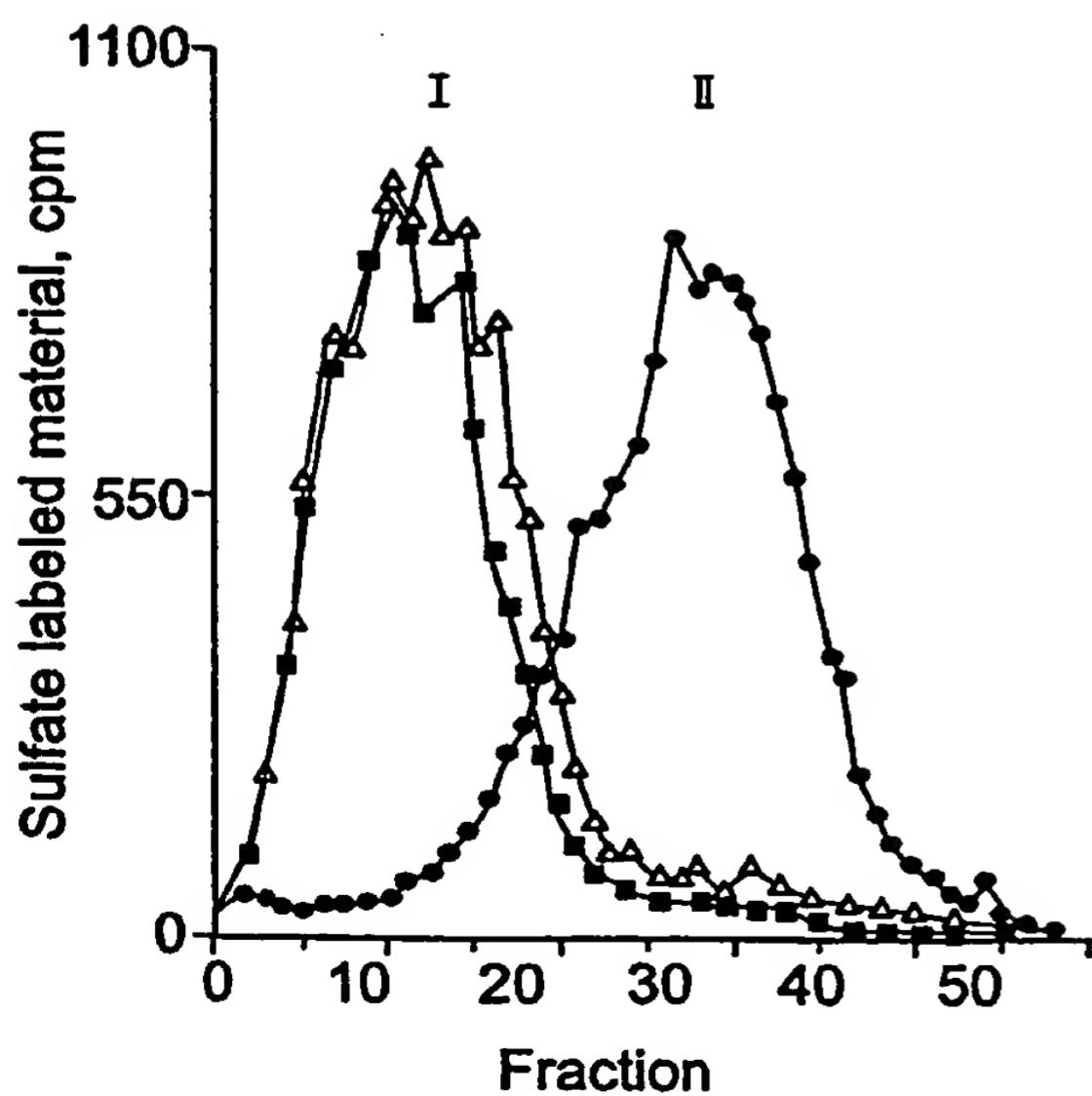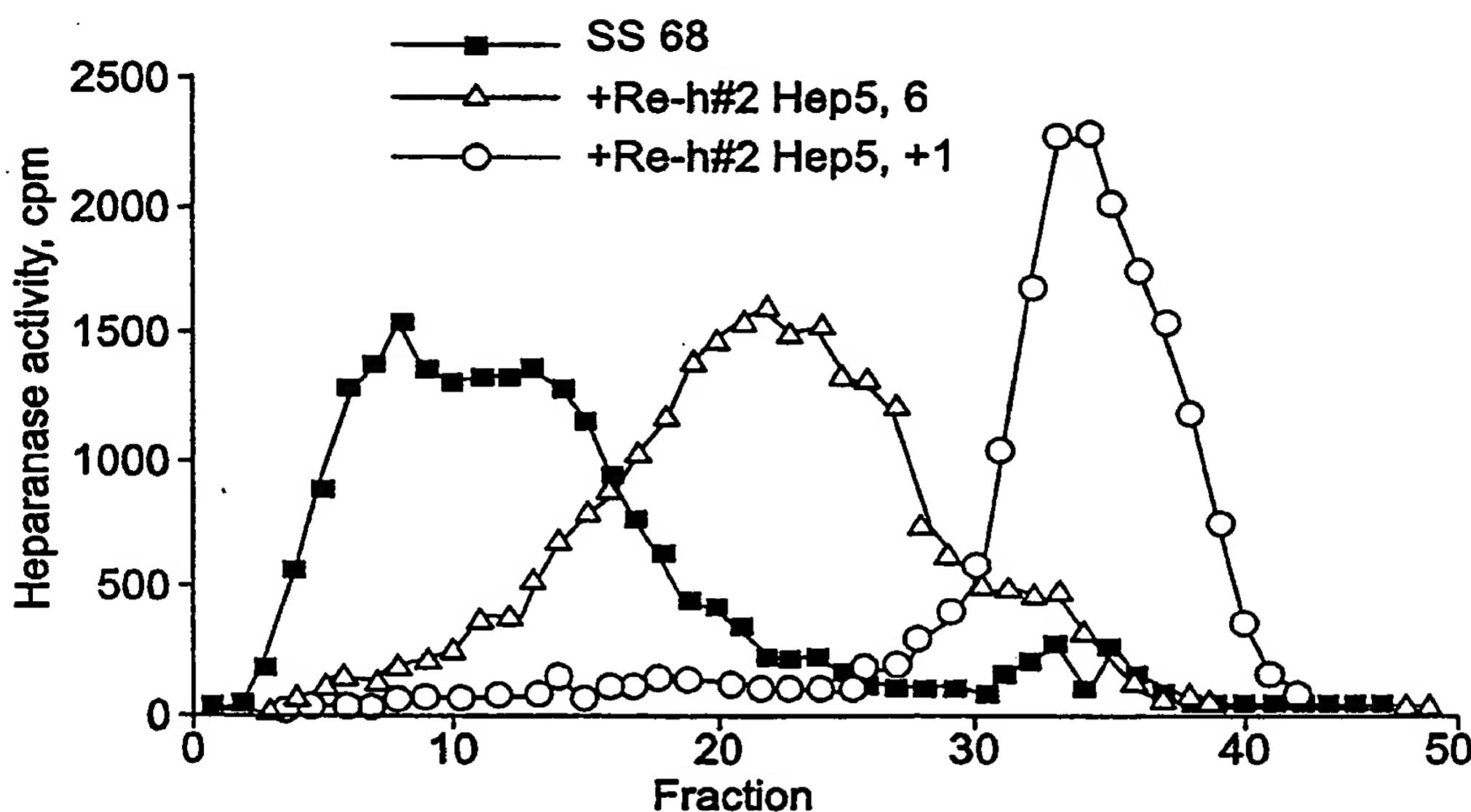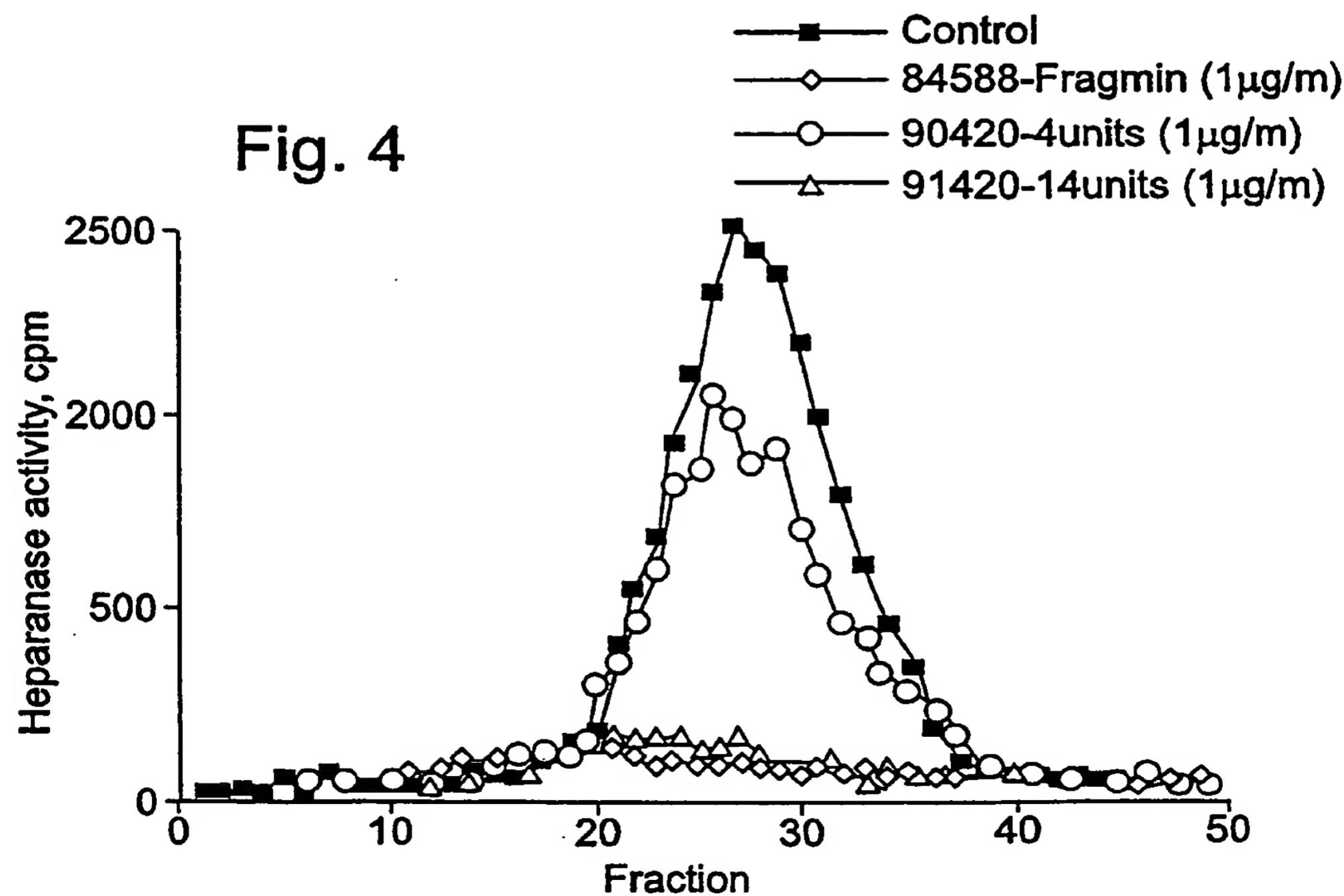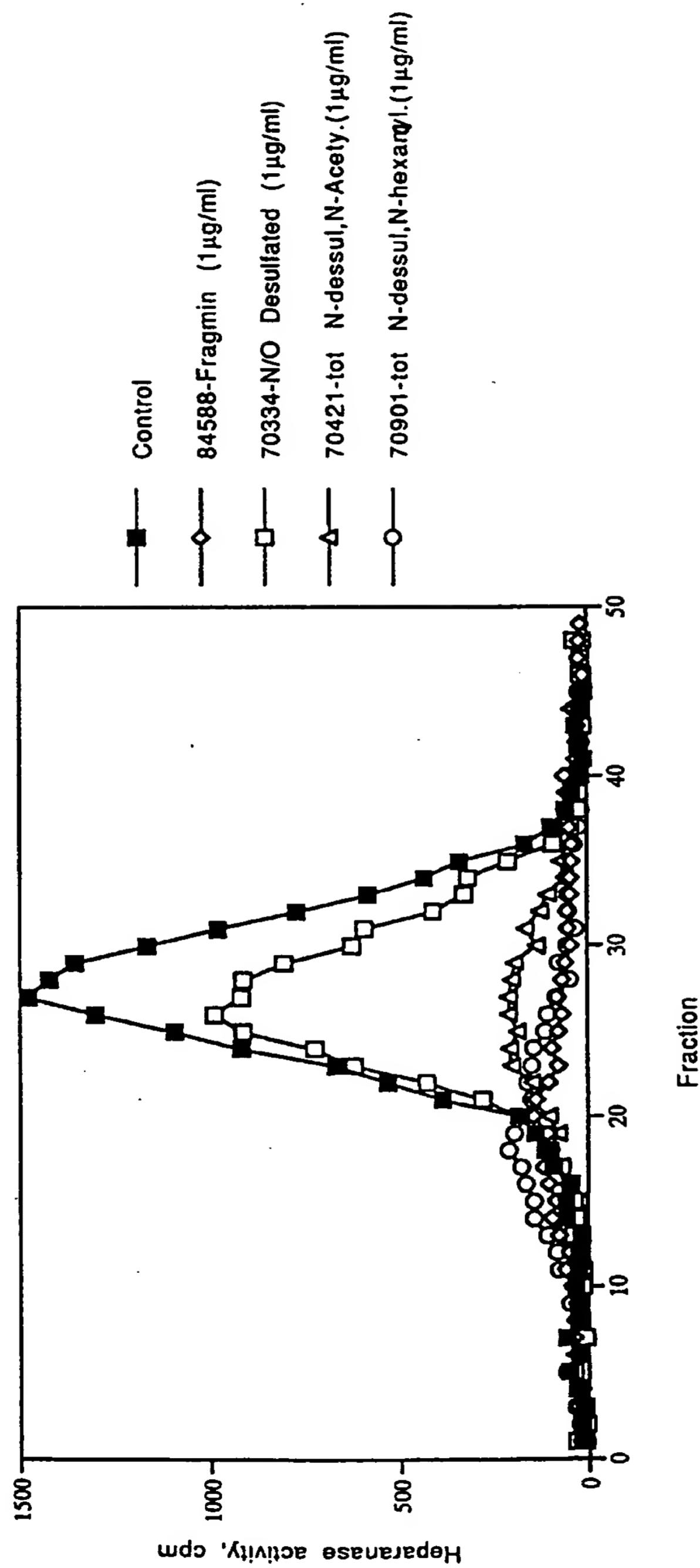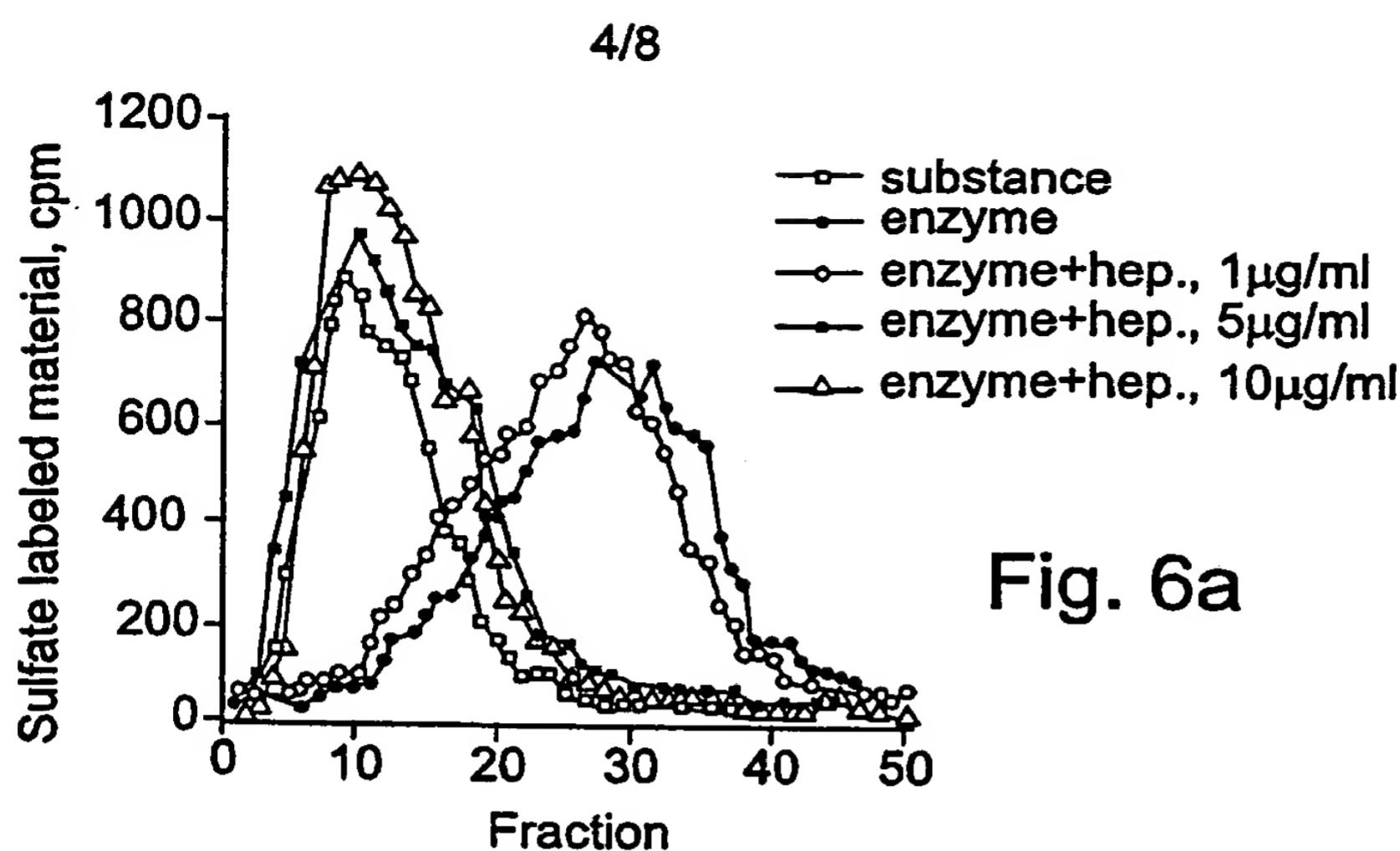
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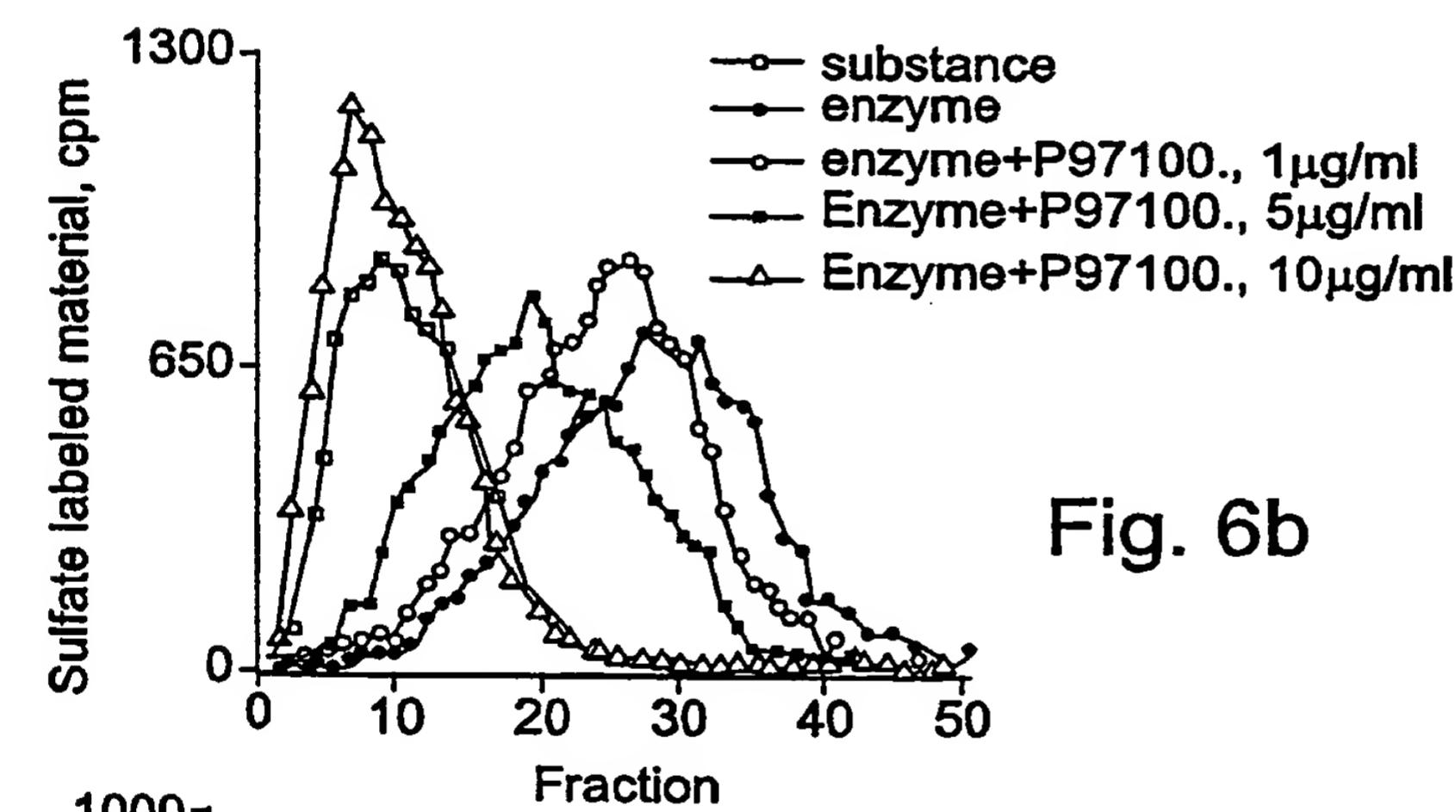
Fig. 6b

Fig. 6c

Fig. 7a

Fig. 7b

Fig. 6

Fig. 9a

Fig. 9b

R²=0.9355

Heparanase (µg)

Heparanase activity (ng glucose equivalent/min)
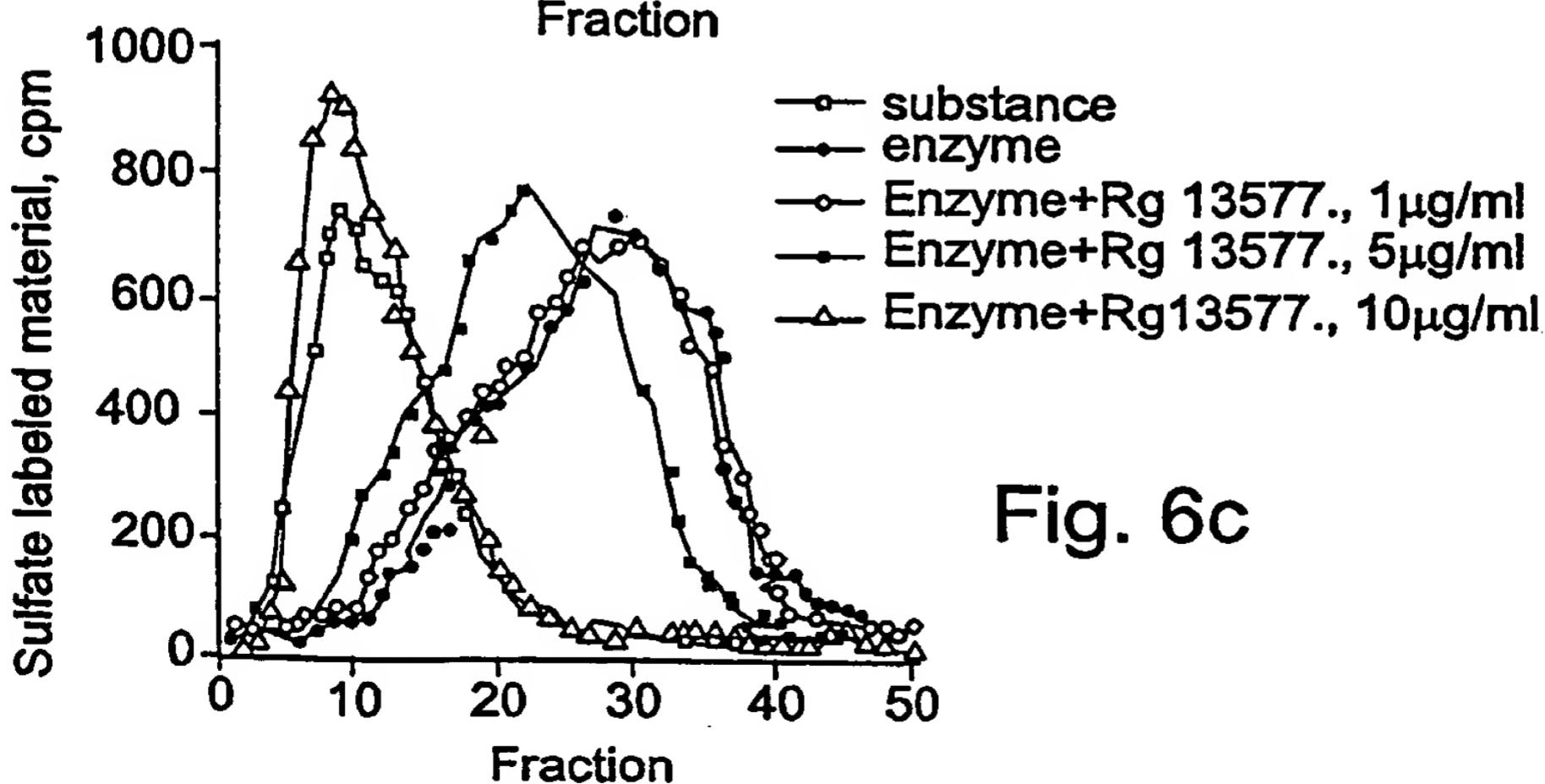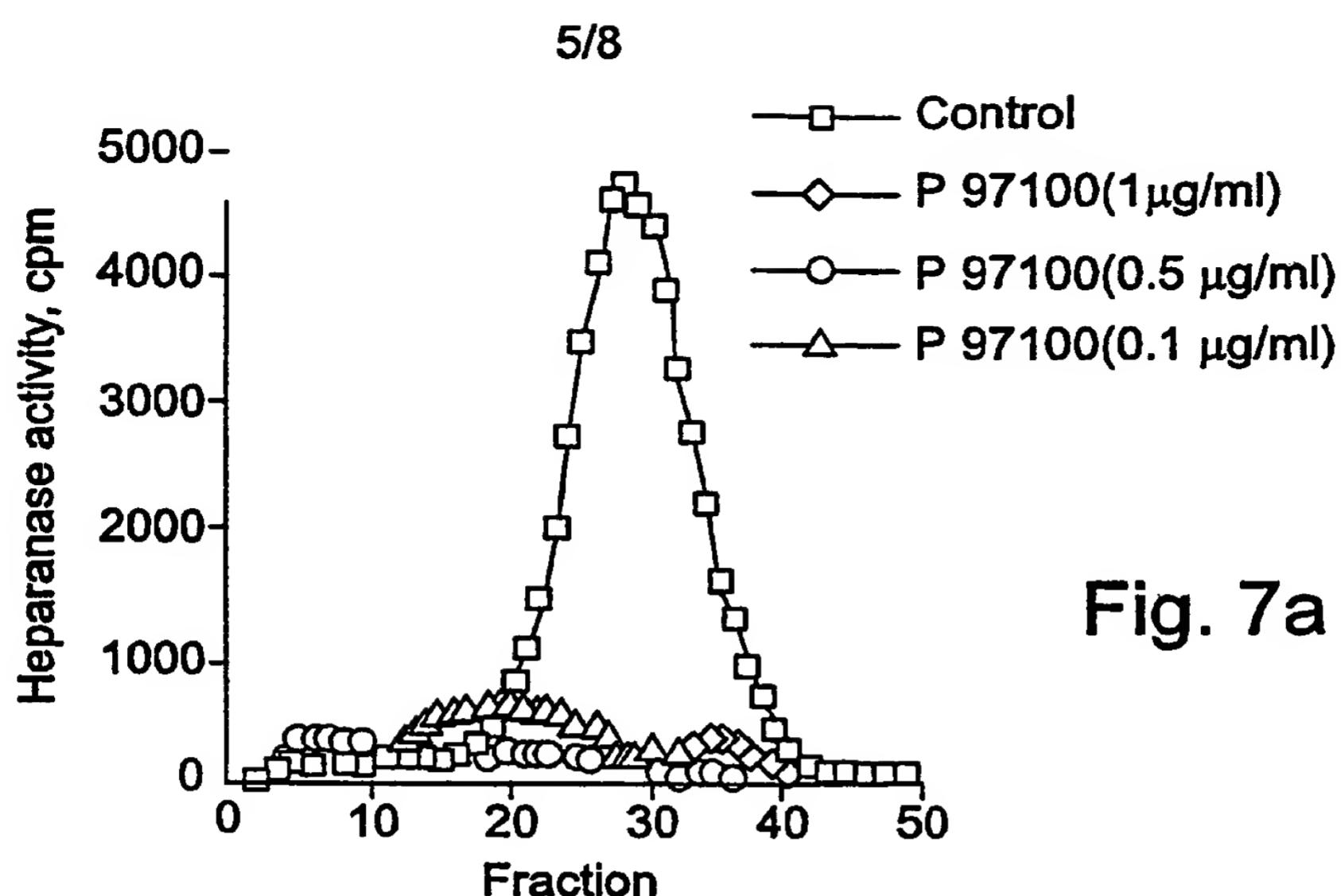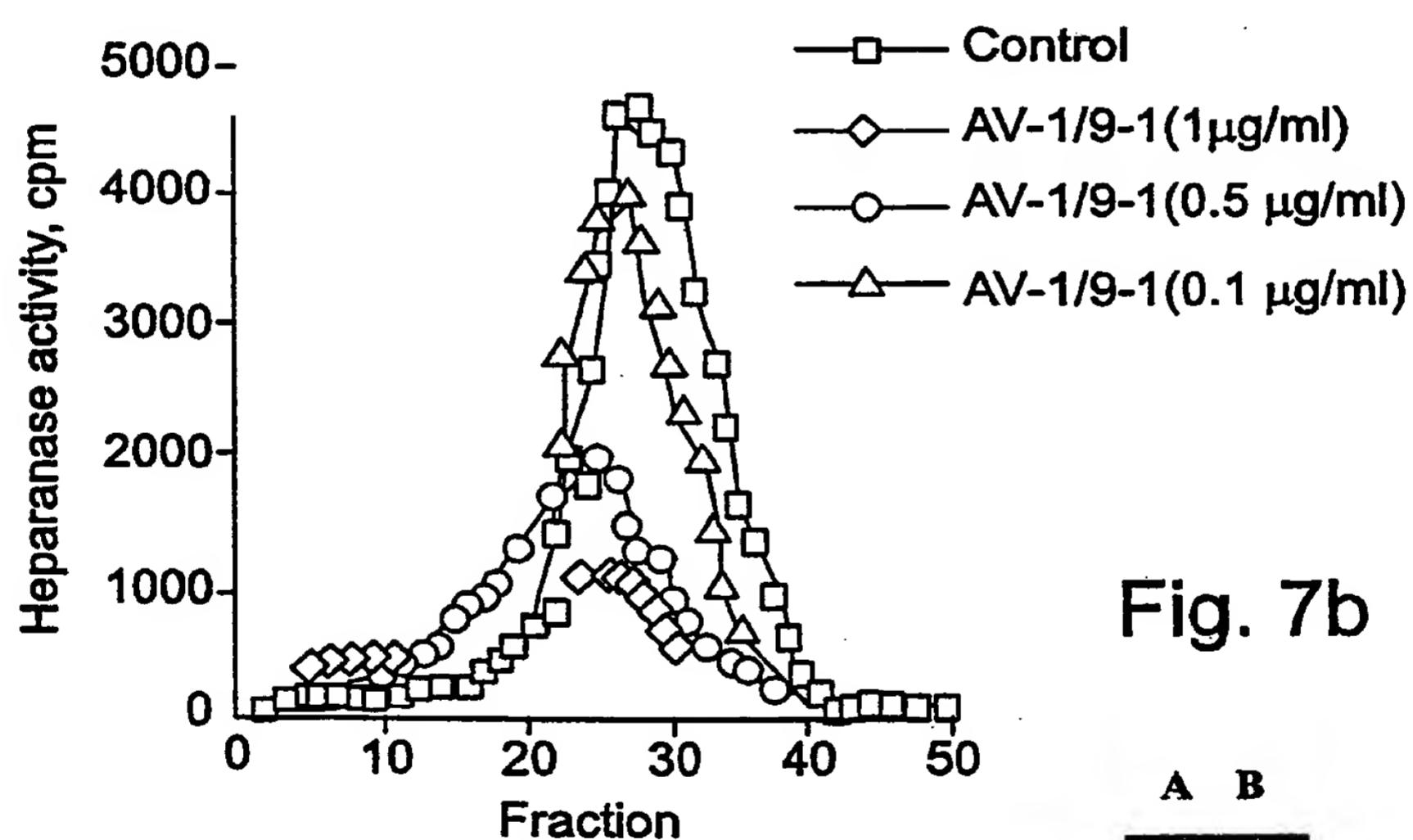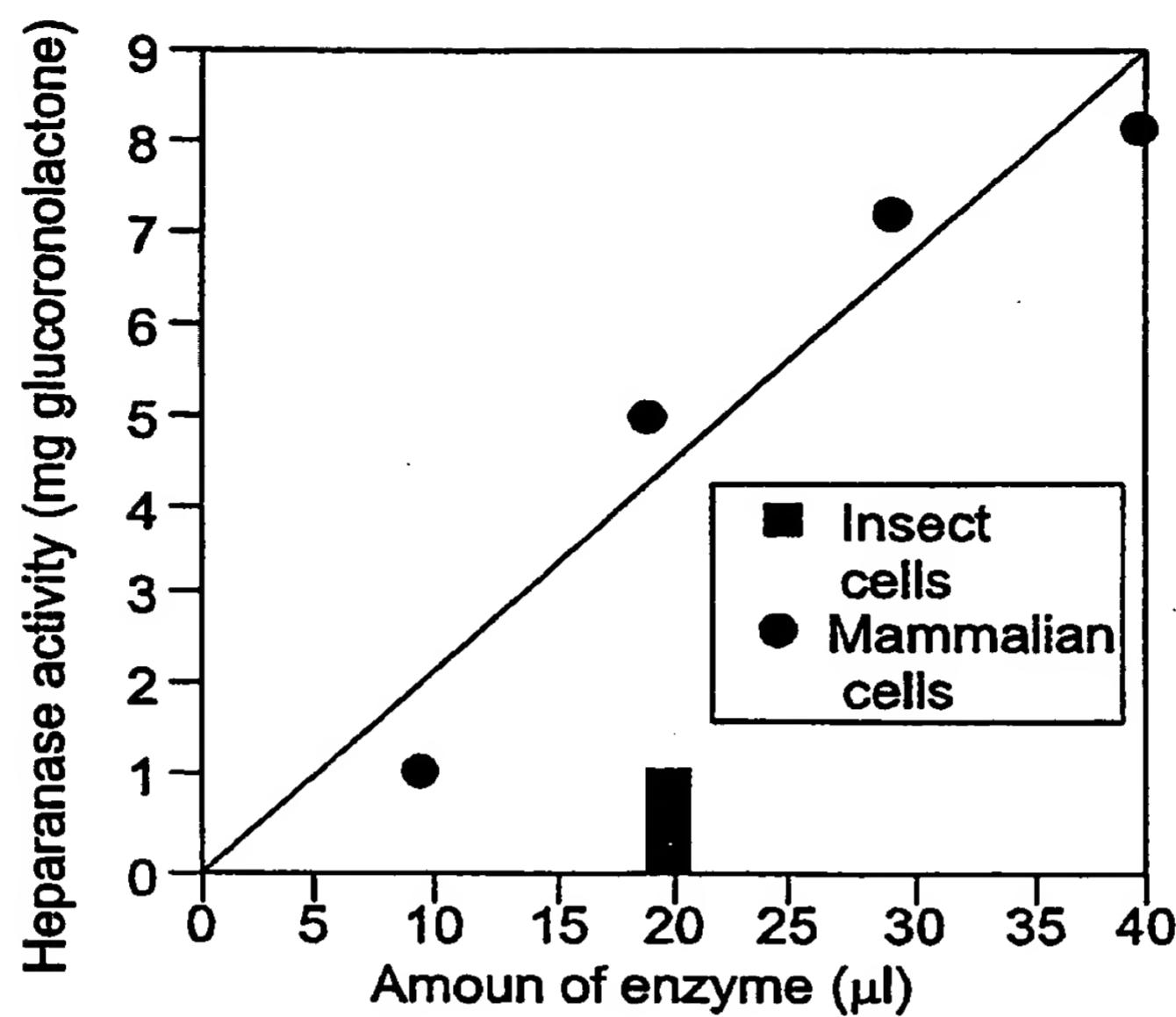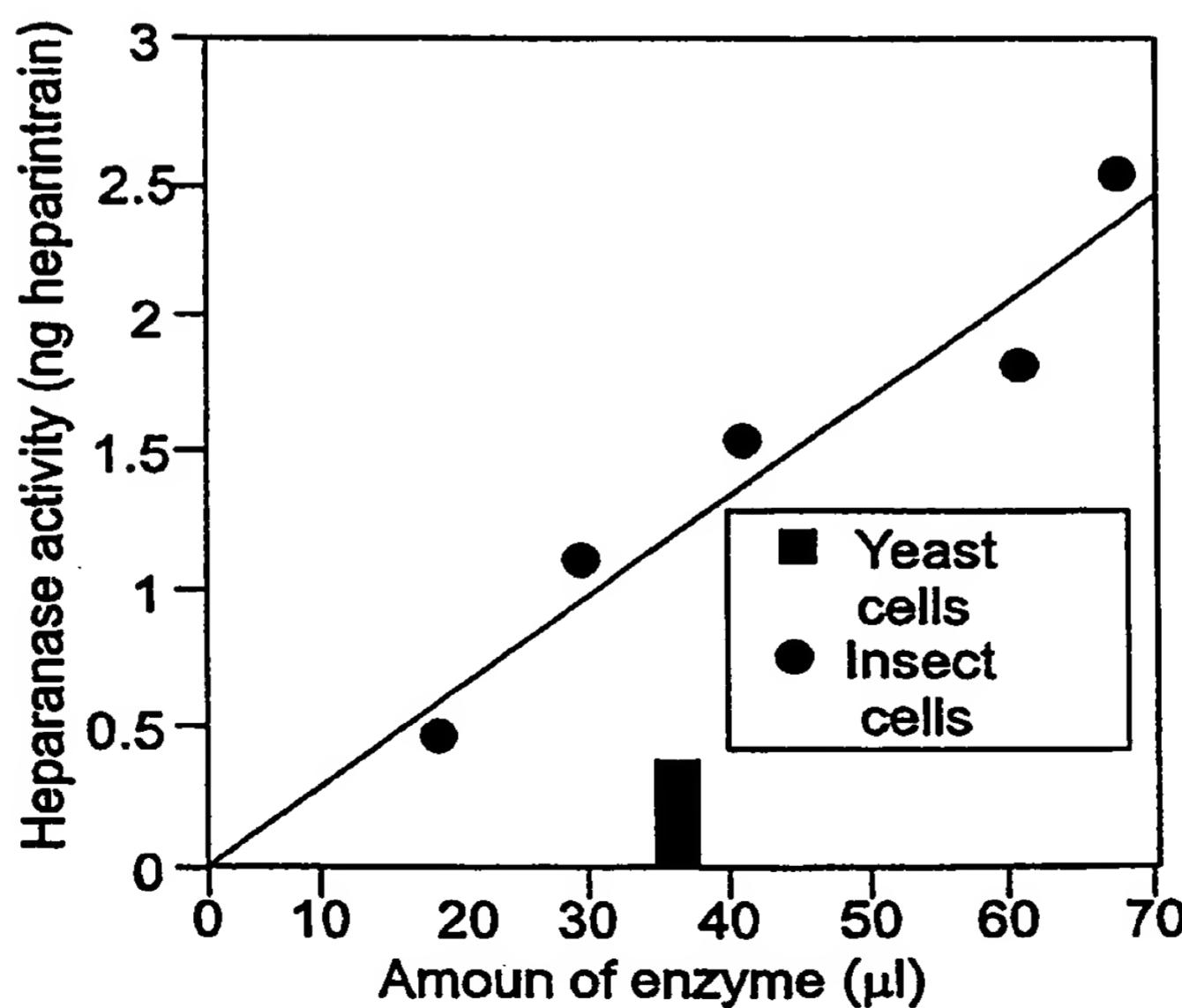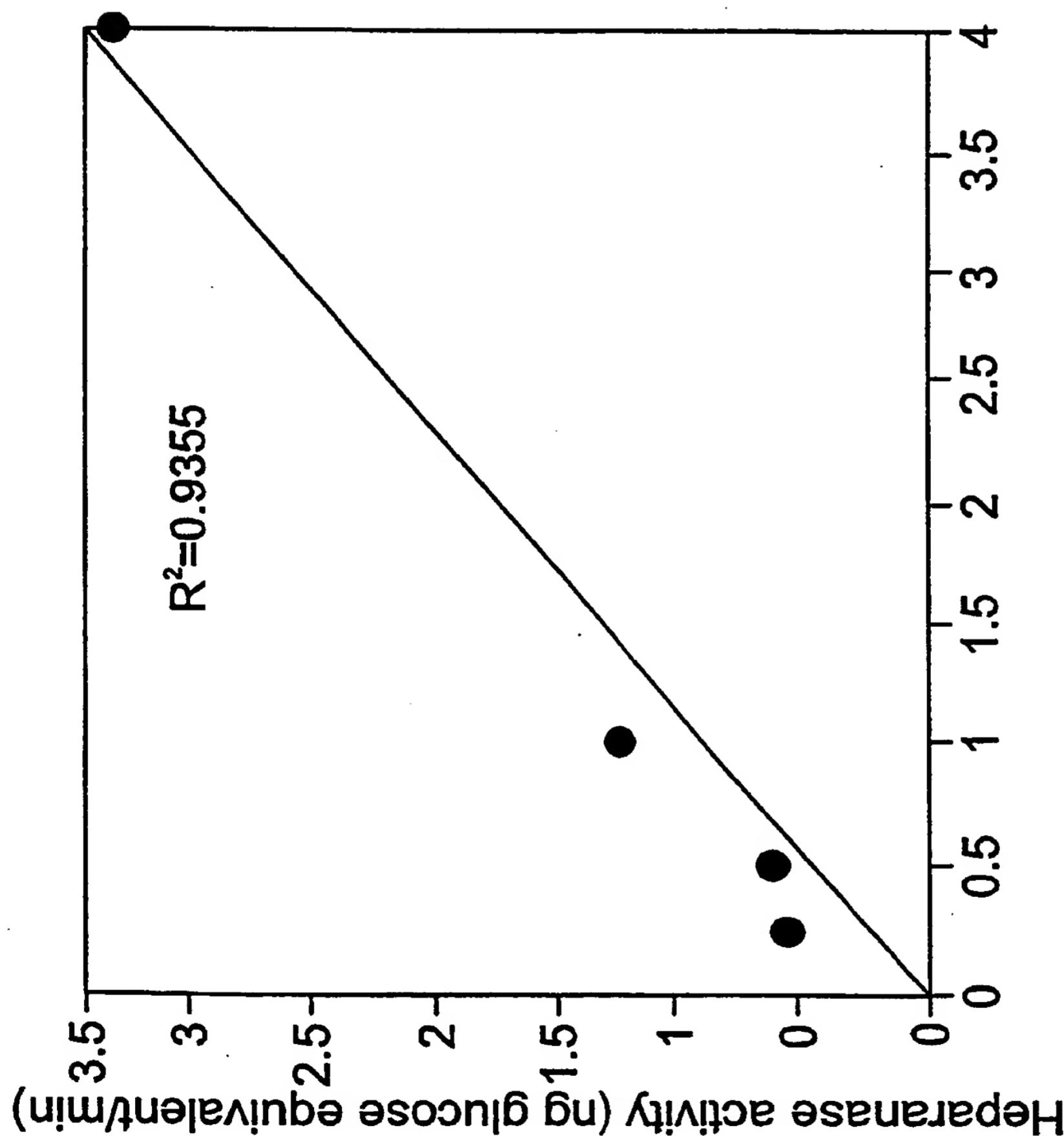
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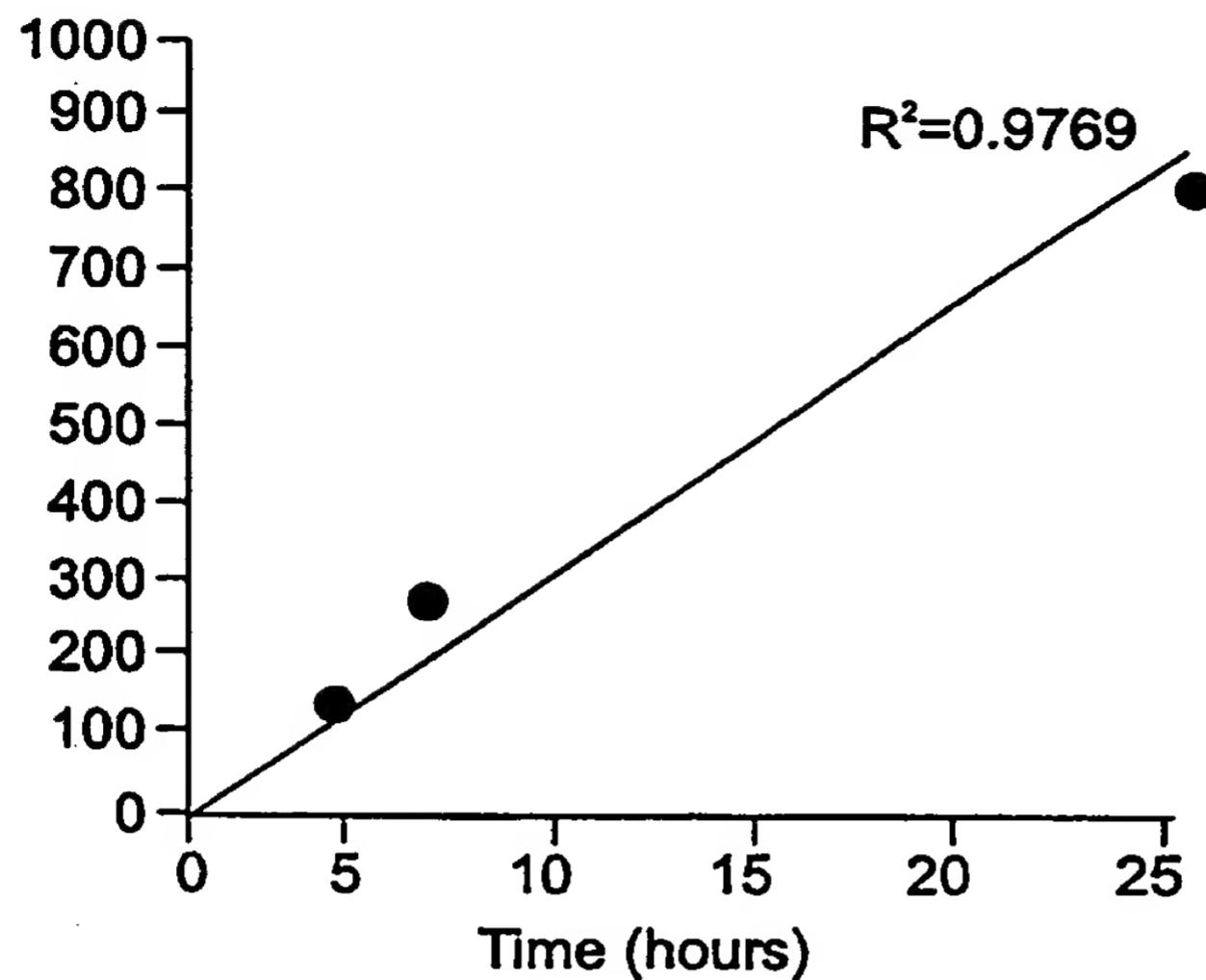
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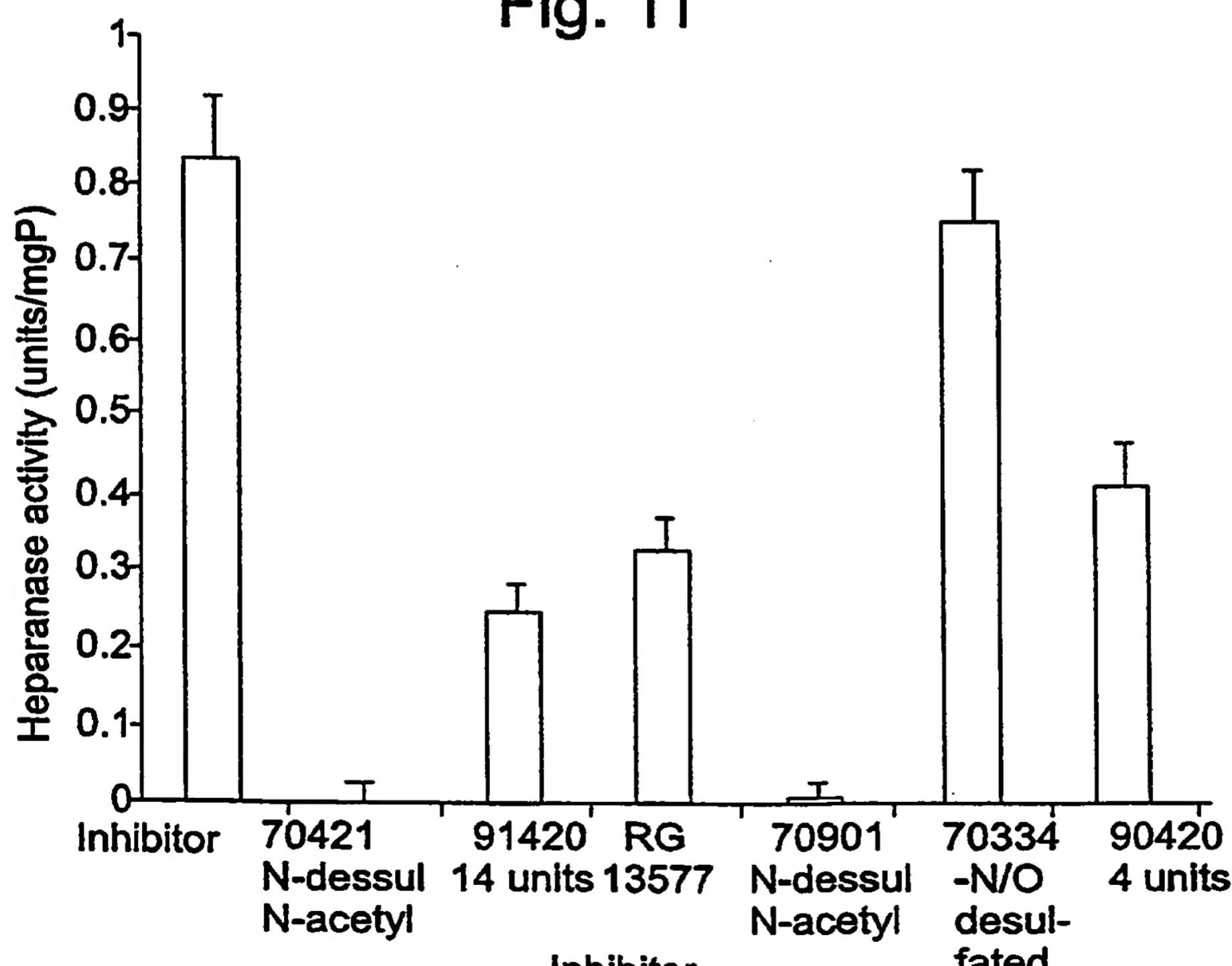
Fig. 12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/15643

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6)   : C12Q 1/34; C12N 9/99, 9/26
US CL :435/18, 184, 201

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/18, 184, 201

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPT, EPOABS, JPOABS, INDEX BIOSCIENCE
search terms: heparanase, carbazole, methylene blue, tetrazolium

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,362,641 A (FUKS et al.) 08 November 1994, see especially column 11, line 45, to column 12, line 56.	92-131 -------- 1-91
X --- Y	US 4,859,581 A (NICHOLSON et al.) 22 August 1989, see entire document.	1-17,20-36,39-56,67-82,90-105,114-128 -------- 18-19,37-3 8,57-66,83-89,106-113,129-131

X	Further documents are listed in the continuation of Box C.		See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 SEPTEMBER 1999	**07 OCT 1999**

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JON P. WEBER, PH.D.
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International application No.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,206,223 A (VLODSAVSKY et al.) 27 April 1993, see especially Table 1.	1-17,20-36,39-56,67-82,90-105,114-128 -------- 18-19,37- 38,57-66,83-89,106-113,129-131
Y	US 5,129,877 A (GALLO et al.) 14 July 1992, see especially column 4, line 9 to column 5, line 21.	18-19,37-3 8,57-66,83-89,106-113,129-131
Y	US 5,399,351 A (LESHCHINER et al.) 21 March 1995, see especially column 9, lines 28-36.	18-19,37-3 8,57-66,83-89,106-113,129-131
Y	US 5,550,116 A (LORMEAU et al.) 27 August 1996, see especially column 17, lines 23-30.	18-19,37-3 8,57-66,83-89,106-113,129-131
Y	US 5,667,501 A (FOWLER et al.) 16 September 1997, see especially column 10, lnes 10-15.	18-19,37-3 8,57-66,83-89,106-113,129-131

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